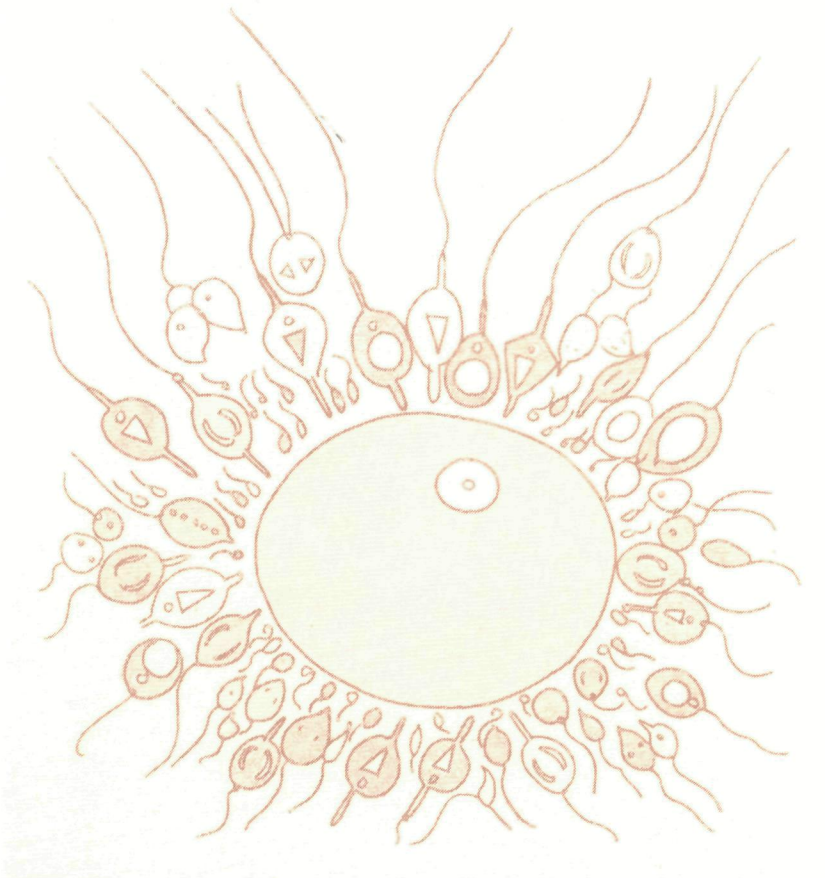
# THE INFERTILE COUPLE:

## SOME CYCLE AND SPERM CHARACTERISTICS

B.P.J. VAN DUREN



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# Voorwoord

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# CHAPTER 1

# INTRODUCTION AND OUTLINE OF THE INVESTIGATION

## 1.1 INTRODUCTION

Infertility is defined as a failure to conceive after at least one year of unprotected intercourse.[1] The common estimation is that around ten percent of the couples are infertile if no treatment is given.[2] It is difficult to estimate the relative contribution of both partners to the infertility. The account to infertility of a female factor may range from 44% to 70%, and of a male factor from 21% to 31% as indicated by different authors.[3-9] Unexplained infertility has been reported to occur with an incidence ranging from 6 to 28%.[10] It is not always a single factor that contributes to the inability to conceive, multifactorial infertility is not uncommon.[4-6]

For different reasons one must realise that the fertility of an individual is relative. Fertility and infertility are not always absolute. There is a gradual difference possible between the two extremes. The interactions between the fertility potentials of each partner determines the ultimate fertility potential of the couple. Furthermore one's (in)fertility is not a constant condition, but may change along with time.

Prior to understanding the problem of infertility, knowledge of the physiological mechanisms involved in the regulation of oocyte maturation, ovulation, sperm transport, fertilisation and implantation is necessary.

## 1.2 GENERAL REVIEW OF THE PHYSIOLOGY OF REPRODUCTION

### 1.2.1 Fertilisation

During coitus approximately 200 x $10^6$ spermatozoa are deposited on the cervix and posterior vaginal fornix. Human semen coagulates immediately following ejaculation, but then becomes liquefied by seminal proteolytic enzymes within a few minutes.[11] After liquefaction the spermatozoa are capable to penetrate the cervical mucus. The coagulum and the buffering capacity of the seminal plasma protect the spermatozoa from the acid environment of the vagina. Cervical secretion also increases the alkalinity which is more favourable for spermatozoa.

Spermatozoa can penetrate cervical mucus by their intrinsic motility, but due to a selection process only morphologically normal sperm cells seem to be able to pass the cervix.[12-16] Roughly one spermatozoon out of thousand is capable of passing the

cervical barrier and ascend into the uterus. Ovarian hormones, especially oestrogens, regulate the secretion and physical properties of cervical mucus. The amount and the quality of cervical mucus show cyclic variations. The condition of cervical mucus greatly influences sperm receptivity. During the preovulatory period the mucus is most receptive to sperm penetration.[17,18]

The second barrier spermatozoa have to overcome before reaching the site of fertilisation is the uterotubal junction. At this point it also seems that only one of every thousand spermatozoa succeeds in reaching the Fallopian tube. Hence, only approximately 200 spermatozoa of the ejaculate arrive into the proximity of the oocyte.[19]

During their time-related passage through the female genital tract, spermatozoa undergo a series of morphological, biochemical and physiological alterations before they acquire the capacity to fertilise.[20] These processes by which the sperm surface is transformed are called capacitation and acrosome reaction. Capacitation is essential for penetration of the zona pellucida and acrosome reaction is a necessary event sperm must undergo to fuse with the oocyte membrane. Capacitation and acrosome reaction can also successfully be completed in appropriately chosen *in vitro* culture conditions.[21-23] The fertilisable life span of human spermatozoa is uncertain but probably around 48-72 hours, although motility can be maintained after the sperm has lost the ability to fertilise.[24-26] The duration of life of the human oocyte ranges between 12 and 24 hours.[27] Thus, fertilisation is only possible two to three days prior to and on the day of ovulation. This is an intrinsic restriction of human fertility, however, the conditions in the female for conception are optimal at this time.

Following ovulation, that is the release of the oocyte from the Graafian follicle of the ovary, the oocyte is picked up by the fimbriated end of the fallopian tube. Most likely the ampulla is the site of fertilisation in the human. The oocyte is surrounded by the zona pellucida, an acellular layer, which becomes impervious to other sperm after fertilisation. The first meiotic division is resumed during ovulation and completed in the oviduct following sperm penetration. One spermatozoon penetrates the zona pellucida, subsequently there is fusion of the vitelline membranes of the oocyte and the spermatozoon. Then the chromatin material of the sperm head decondenses and the male pronucleus is formed. After the male and female pronuclei have been fused, fertilisation is accomplished, and cleavage starts while the ovum is being transported to the uterus. The fertilised ovum implants in the uterus around six to seven days after fertilisation.[28] By then, it has developed to the stage of a blastocyst.

The human conceptus produces human chorionic gonadotrophin (hCG) which can be measured in the maternal plasma at approximately day eight of pregnancy.[29,30] The function of the corpus luteum is crucial during the first stage of pregnancy and optimal hormonal conditions are necessary for implantation. It is difficult to estimate the true incidence of fertilisation and implantation. It is assumed that circa 50% of the fertilised ova and embryos are lost during the luteal phase without a delay of menstruation and this phenomenon is known as early conceptual loss.[31]

### 1.2.2 Hormonal pattern of the menstrual cycle

The menstrual cycle upon which fertility depends is characterised by a complex but orderly sequence of endocrinological and morphologic events. Complex interrelationships exist between the hypothalamus, the anterior pituitary and the ovaries. The menstrual cycle is a consequence of the integration of the signals of these three components into a control system with negative and positive endocrine feedback mechanisms as well as neural inputs from higher central nervous system centers. (for reviews see e.g. 32-35)

The hypothalamus produces gonadotrophin releasing hormone (GnRH) which is released into the portal circulation in an intermittent or pulsatile way. Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH) are synthesised and secreted in the anterior pituitary in response to GnRH and are also released in bursts. Oestrogens from the ovaries have an important modulating effect on the pituitary and the hypothalamus. The physiology of the normal cycle can at best be described by dividing the cycle into three major phases: the follicular phase, the ovulatory phase and the luteal phase.

Follicular growth is a continuous autonomous process which lasts from fetal life until the menopause, but without adequate gonadotrophic stimulation the follicles become atretic in an early stage.

At the end of a cycle when the corpus luteum regresses and progesterone and 17ß-oestradiol levels decline, the negative feedback of these steroids on FSH levels is low. The rise in FSH levels rescues a group of follicles from atresia. These primordial follicles consist of an oocyte surrounded by granulosa cells. FSH stimulates the aromatisation of androgens to 17ß-oestradiol in the granulosa cells and 17ß-oestradiol levels increase progressively. Both FSH and oestradiol have a stimulatory effect on the proliferation of the granulosa cells. Through negative feedback of oestradiol the secretion of FSH declines. The rise in the secretion of oestradiol has a negative feedback effect on the hypothalamus and pituitary and prevents other follicles to develop. The androgen precursors of oestradiol, mainly androstenedione, are produced by the cells of the theca interna and the stromal tissue under the stimulatory effect of LH. The plasma level of LH slowly increases. An androgenic milieu and a prematurely elevated LH level inhibit the granulosa cell proliferation and cause degenerative changes in the oocyte. By the sustained accumulation of granulosa cells the primordial follicle changes into a preantral follicle and subsequently into an antral follicle which contains follicular fluid. Selection of the dominant preovulatory follicle has occurred by the middle of the proliferative phase.[36]

At high concentrations such as in the late follicular phase, the negative feedback of oestradiol changes into a positive feedback resulting in a surge of LH and FSH.[37] The oestradiol concentration is maximal 24 - 36 hours prior to ovulation and drops after the LH surge. The rising levels of LH in the late follicular phase cause luteinisation of the granulosa cells with increased production of progesterone prior to the LH peak and ovulation. This results in slightly increased serum progesterone levels just prior to the

onset of the LH surge. There is some evidence that progesterone may be essential for inducing the preovulatory surge of FSH and that it facilitates the LH release at mid-cycle.[38] Approximately 28 - 32 hours after the onset of the LH surge and 10 to 12 hours after the LH peak the oocyte is released from the follicle, i.e. ovulation, and the follicle is transformed into a corpus luteum.

Normal luteal function requires optimal preovulatory follicular development. During the luteal phase progesterone in the serum increases steadily to maximum levels which are reached at circa six to eight days after the LH peak. The corpus luteum produces also oestradiol, 17α-hydroxy-progesterone and androgenic steroids. The oestradiol concentration also rises in the luteal phase but the maximum concentration is only one third of the preovulatory peak. Through negative feedback of progesterone and oestradiol on LH and FSH production the growth of new follicles is inhibited. Unless pregnancy occurs regression of the corpus luteum starts circa 10 days after ovulation and the steroid levels decline. Subsequently the FSH level increases and a new menstrual cycle is initiated.

The physiological role of nonsteroidal factors in the control of the menstrual cycle has not fully been established as yet. Whether inhibin, a nonsteroidal regulator in follicular fluid, is a normal component of feedback regulation is unknown. It is supposed selectively to suppress the secretion of FSH from the pituitary gland.[39]

### 1.2.3 Androgenic steroids

In the female androgenic hormones originate from the adrenal cortex, from the stromal tissue, theca interna and corpus luteum of the ovaries, and from peripheral conversion of other androgenic steroid precursors. (for reviews see 40-42)

The androgens most extensively studied are testosterone, 5α-dihydrotestosterone (DHT), androstenedione, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS). DHEA and DHEAS are secreted primarily by the adrenal glands: 80% and 90%, respectively.[40] Approximately 50% of testosterone levels arise from peripheral conversion of androstenedione, while the adrenal and ovary contribute equally to the remaining 50% of the circulating levels of testosterone. Androstenedione is secreted directly by both the adrenals and ovaries in around equal amounts.[41]

The secretion of androgens is controlled by the hypothalamus and the pituitary. Adrenal androgen production is stimulated by the adrenocorticotrophic hormone (ACTH) and probably by other factors.[43] Prolactin has also been considered to stimulate adrenal androgen secretion, by potentiating the effect of ACTH on adrenal androgen release.[43,44] Prolactin may also play a role in peripheral androgen metabolism.[44] However, Wathen et al.[46] found an inverse relationship between prolactin and both DHEAS and testosterone. The production of ovarian androgens is stimulated by the gonadotrophins, mainly LH.[47] In the ovary androgens are the precursors of oestrogens. In the granulosa cells androstenedione is aromatised to oestradiol.[48]

The intrinsic biological activity of the androgens is different: testosterone and DHT show a very strong androgenic activity, whereas DHEA and DHEAS are less potent androgens.[49]

Most androgens are bound to proteins in plasma.[50] Circa 80% of the circulating testosterone is bound to sex hormone binding globulin (SHBG).[51] This carrier protein has also an affinity for other androgens and oestrogens. Furthermore, the production of SHBG is decreased by androgens and increased by oestrogens. Approximately 19% of testosterone is bound to albumin, whereas only 1% is unbound and free. Only the unbound proportion of a hormone is able to exert biological activity. Most assays of serum testosterone measure the total testosterone concentration, i.e. both the amounts of protein-bound and -unbound. DHEA, DHEAS and androstenedione are not significantly protein-bound. Measurement of androgenic steroids in saliva are reported to correlate well with the concentrations of nonprotein-bound hormones in blood.[52,53]

Androstenedione, testosterone and DHEA all show diurnal changes similar to that of cortisol.[54] Some studies also show androstenedione and testosterone to exhibit cyclical patterns. During the periovulatory period the levels of these androgens are elevated due to increased ovarian production, but variations in ovarian androgen secretion can be masked by fluctuations in adrenal secretion.[40,41]

## 1.3 THE GENERAL FERTILITY WORKUP PROGRAM

The burden of the investigation and treatment of infertility for the patient should not be underestimated. In the first place a fertility workup program should be systematic and the most easily performed, non-invasive tests must be carried out first. The basic fertility survey should be performed as rapidly as possible. Furthermore the infertility investigation has to be complete as infertility often can be multifactorial in origin. The investigation and management of infertility must be handled with care as it may affect the couple's sexual life and social function. Accurate information and emotional support by the physician may diminish the psychological strain.

The main items of a fertility workup program are:[10,55]

- Medical history
- General physical and pelvic examination
- Basal body temperature (BBT) records with hormonal assays if necessary
- Semen analysis (SA)
- Examination of sperm-cervical mucus interaction
- Tests of tubal function

The initial assessment of the infertile couple should begin with a thorough and systematic medical history and physical examination of both partners. The first visit also gives the opportunity to inform the couple about the examinations and tests that will

follow. If no gross abnormalities become apparent the next steps should be BBT charts for the wife and semen analyses for the husband.

The recording of the basal body temperature gives indirect information on the occurrence and time of ovulation. The pattern throughout the cycle rather than the absolute temperature values is important in the interpretation of the BBT record. However, when correlated with endocrinological or ultrasonographical markers the BBT is not always reliable.[56-58] The BBT chart is helpful when it is clearly biphasic. When the pattern is doubtful or when the exact day of ovulation must be detected additional information is needed. Additional cycle evaluation can be obtained by measurement of reproductive hormones enabling the diagnosis of hyperprolactinemia, and/or hyperandrogenism. Follicular growth and the occurence of ovulation can be detected by ultrasound.

Semen analysis is the most important test in the evaluation of male fertility and must be carried out at least twice at two different occasions with an interval of at least six weeks. It is a quantitative test which gives information on number, motility and morphology of the spermatozoa, in addition to several characteristics of seminal fluid parameters. A functional test in the evaluation of male fertility can be the zona-free hamster ovum sperm penetration assay (SPA).[59] This assay determines the capability of human spermatozoa to penetrate hamster oocytes from which the cumulus and the zona pellucida have been removed. The spermatozoa have to be preincubated to capacitate.

It is a relatively laborious test to perform, while its indications and clinical significance is still subject of discussion.[60,61]

The next step is the evaluation of the sperm-mucus interaction. The postcoital test is scheduled just prior to ovulation. Within 8 the 12 hours after intercourse, some mucus is obtained from the cervical canal and viewed under the microscope (magnification 400x). The quality of the mucus is determined and expressed in terms of amount, "Spinnbarkeit", ferning and clarity.[62,63] The quality of the cervical mucus is an indirect indicator of relative levels of oestradiol and progesterone: oestradiol stimulates its quantity and properties whereas progesterone has an opposite effect.[17] If the poor mucus quality is related to inaccurate timing, the postcoital test should be repeated. The test is judged positive if at least five spermatozoa with good forward progression per field can be seen at 400x magnification. If the postcoital test is negative despite good scheduling, another test -the sperm penetration meter (SPM) test- gives the opportunity to observe the sperm-mucus interaction *in vitro*. *In vitro* testing by applying donor mucus or donor sperm can be an aid to determine whether the poor postcoital test is either due to factors in the mucus or to intrinsic defects in the sperm.[64,65]

Sperm antibody testing should be performed in all cases of agglutination of the spermatozoa.[66]

Usually the last and most invasive step in a basic fertility survey is testing of tubal patency. A hysterosalpingogram images the inner contours of the uterine cavity and the patency of the Fallopian tubes. The way in which the contrast medium spreads into the peritoneal cavity may provide information on tubo-peritoneal pathology. By laparoscopy the inner pelvic organs can be visualised and inspected. Adhaesions and endome-

triosis can be detected and tubal patency can be tested by chromepertubation of the Fallopian tubes.

The investigation of an infertile couple should be systematic, rational and directed towards the individual problems.

## 1.4 OUTLINE OF THE PRESENT INVESTIGATION

The purpose of the present study was to investigate the processes of oocyte maturation, ovulation and corpus luteum function in the female and to determine how these processes could be assessed in an optimal and rational manner in a fertility workup program. Since there is evidence that a number of pregnancies are lost previous to the menstrual period, the phenomenon of early conceptual loss in the luteal phase was studied in addition to the corpus luteum function as a possible cause of unexplained infertility.

Furthermore the value of newly developed diagnostic methods to determine male fertility was assessed.

To elucidate these problems the investigation was divided in three parts:

I: A prospective, extensive study of the menstrual cycle in infertile and assumed fertile women took place. Their ovarian function was assessed by frequent sampling of blood and saliva in which the full range of relevant hormone determinations were performed. Three study groups were investigated: a group of women with irregular cycles and women of couples suffering from unexplained infertility with apparently normal cycles were both compared with a control group of normally cyclic women.

II: The problem of early conceptual loss was studied prospectively. Frequent determinations of hCG took place during the luteal phase of two groups of women. Twenty infertile women of couples with unexplained infertility formed the study group and 20 normally fertile women who were trying to conceive formed the control group.

III: The value of the SPA, a functional test of the fertilising capacity of human spermatozoa was evaluated by comparing this test with the routine semen analysis, the SPM test and IVF with special attention to the post-capacitation motility.

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# CHAPTER 2

# SUBJECTS, MATERIALS AND METHODS

## 2.1 INTRODUCTION

Three groups of patients were selected to participate in an extensive fertility workup program. Twenty women with proven or assumed normal fertility formed the control group. Thirty-five infertile women who attended a gynaecologist for their infertility participated also in the investigation. These patients were divided into two study groups: fifteen patients with irregular menstrual cycles and twenty patients with apparently normal menstrual cycles, but suffering from unexplained infertility.

An extensive study of endocrine parameters of the menstrual cycles of these three groups took place. Blood and saliva samples were collected at regular intervals throughout one cycle. Twelve endocrine parameters were determined in the serum samples and eight hormone determinations took place in the saliva samples.

The results of the group with irregular cycles vs. the control group and of the group with unexplained infertility vs. the control group were evaluated.

## 2.2 SELECTION OF SUBJECTS

### 2.2.1 Selection criteria for the control group

Twenty healthy women were willing to participate in the investigation as a volunteer. They were attending a gynaecologist for a problem not interfering with the investigation of their menstrual cycle, e.g. candidates for laparoscopic tubal obstruction or an intrauterine device. Some women of the control group were hospital workers or students and the remaining women were recruited by means of an advertisement in a local paper.

The following criteria were used for admittance into the control group:

- A history of regular menstrual cycles, as a rule ranging in length from 26 - 32 days, during the last three months;
- Younger than 38 years;
- No hormonal or other medication;
- No pregnancy or lactation period during six months preceding the investigation;
- No obesity, as indicated by the Quetelet-index (see 2.3);
- Good general health.

### 2.2.2 Selection criteria for the infertile women with irregular cycles

The group of women with abnormal menstrual cycles comprised fifteen women with a complaint of infertility of at least one year. They had repeatedly irregular cycles: i.e. menstrual intervals of less than 26 days or between 32 and 90 days, whether ovulatory or anovulatory as judged by previous Basal Body Temperature (BBT) charts.

### 2.2.3 Selection criteria for the women with unexplained infertility

The following conditions had to be fulfilled for the women suffering from unexplained infertility:

- Duration of infertility for at least one year;
- Regular, ovulatory menstrual cycles, confirmed by BBT charts, with menstrual intervals as a rule between 26 and 32 days;
- Normal semen analysis of the partner on at least two occasions. (Sperm count ≥20 x $10^6$ /mL, ≥50% of the spermatozoa with good forward progression and ≥60% morphologically normal spermatozoa). Despite normal semen analyses before admittance in the investigation, some men showed one or more abnormal semen parameters during the study;
- Positive postcoital test, i.e. at least five spermatozoa with good forward progression per field, magnification 400 x;
- Tubal patency, no adhaesions and no endometriosis.

## 2.3 CLINICAL CHARACTERISTICS OF THE SUBJECTS

Table II.1 presents details concerning age, height and weight of the subjects. The Quetelet index[1] was used to determine the relative body weight. This index can be calculated from the formula:

$$\frac{\text{body weight}}{\text{height}^2} \left( \frac{\text{kg}}{\text{cm}^2} \times 10^5 \right)$$

The age, height and weight distributions of the women of the three groups were comparable. All women had a Quetelet-index in the expected normal range for their age.[2]
The control group comprised nine women who had never been pregnant and eleven women with one or more children and/or one or more abortions. In the control group the length of the study cycle ranged from 24 to 36 days, despite previous cycle lengths of 26 to 32 days. Two women had short cycles of 24 and 25 days, respectively, and three women had cycle lengths of 33, 35 and 36 days, respectively, during the investigation.

**Table II.1** Age, height, weight and Quetelet-index of the investigated groups

| | n | Mean | SD | Range Min. | Range Max. | Median |
|---|---|---|---|---|---|---|
| Age (years) | | | | | | |
| control group | 20 | 27.8 | 4.8 | 19 | 37 | 29 |
| irregular cycles | 15 | 28.6 | 3.1 | 22 | 33 | 28 |
| unexpl. infert. | 20 | 27.9 | 3.7 | 20 | 34 | 28 |
| Height (cm) | | | | | | |
| control group | 20 | 167.8 | 8.1 | 151 | 180 | 169 |
| irregular cycles | 15 | 166.9 | 5.9 | 156 | 176 | 168 |
| unexpl. infert. | 20 | 166.9 | 5.7 | 158 | 178 | 167 |
| Weight (kg) | | | | | | |
| control group | 20 | 59.7 | 7.4 | 50 | 79 | 58 |
| irregular cycles | 15 | 58.7 | 6.0 | 49 | 71 | 57 |
| unexpl. infert. | 20 | 59.5 | 7.7 | 44 | 79 | 59 |
| Quetelet-index ($\frac{kg}{cm^2} \times 10^5$) | | | | | | |
| control group | 20 | 211.7 | 18.9 | 186.1 | 258.0 | 206.4 |
| irregular cycles | 15 | 210.5 | 17.8 | 177.8 | 235.9 | 212.6 |
| unexpl. infert. | 20 | 213.8 | 27.4 | 161.6 | 267.0 | 208.0 |

Due to cycle variation this could be expected. Additionally, other investigators consider cycle lengths of 24 or more days, but not longer than 36 days as normal.[3]

The median length of the study cycle of the women with irregular cycles was 35 days (range 26 - 49 days). In the group with unexplained infertility the median duration of the investigated cycle was 27 days. Three women had a cycle length of 24 days during the investigation and in two women the study cycle lasted 39 and 63 days, respectively.

Table II.2 gives information on the length of the follicular phase, defined as the interval in days between the first day of menstruation up to and including the day of the LH peak and on the length of the lutcal phase, defined as the interval following but not including the day of the LH peak until the day preceding next menstruation. When presenting the hormonal data in chapter 3 and 4 also a peri-ovulatory phase is introduced including the day of the LH peak, the previous 2 days and the day after the LH peak.

Of the fifteen women with irregular cycles ten suffered from primary infertility and five were secondary infertile. In the group of unexplained infertility two women had been pregnant at least once, the remaining 18 women never had achieved a pregnancy.

The distribution of the duration of the infertility was not significantly different for the

**Table II.2** Cycle lengths and lengths of the follicular and luteal phases of the investigated menstrual cycles of the three study groups

| | | Median | Range | Mean |
|---|---|---|---|---|
| Cycle | Control | 29 | 24-36 | 29.0 |
| Length | Irregular | 35 | 26-49 | 34.4 |
| (days) | Unexplained | 27 | 24-63 | 29.6 |
| Follicular | Control | 17 | 12-23 | 15.8 |
| Phase | Irregular | 19 | 14-35 | 19.6 |
| (days) | Unexplained | 13 | 10-49 | 15.8 |
| Luteal | Control | 13 | 10-16 | 13.2 |
| Phase | Irregular | 14 | 10-17 | 14.5 |
| (days) | Unexplained | 14 | 11-17 | 13.8 |

two infertile groups (median 30 vs 35 months). The longest duration of infertility was six years in both groups.

In both infertile groups seven women visited the outpatient department for a second opinion of their infertility problem after having been investigated and/or treated elsewhere.

All women were examined for their general health. Anaemia was excluded by measuring haemoglobin levels. Liver, renal and thyroid function parameters were determined to assure normal function of the most important organs which can interfere with the function of the hypothalamic-pituitary-ovarian axis. The median values and ranges of some non-hormonal data and thyroid function are presented in table II.3.

**Table II.3** Concentrations of non-hormonal and thyroxine determinations in serum. Median (range)

| | Control | Irregular cycles | Unexplained Infertility | Reference Values |
|---|---|---|---|---|
| Hb (mmol/L) | 8.4 (7.7-9.4) | 8.2 (7.5-9.4) | 8.4 (7.2-9.2) | 8.0-9.5 |
| Urea (mmol/L) | 4.5 (2.1-5.9) | 4.4 (1.4-5.6) | 3.7 (2.3-6.1) | 2.5-5.8 |
| Creatinine (µmol/L) | 62 (54-78) | 65 (53-77) | 64 (53-73) | 53-80 |
| Uric acid (mmol/L) | 0.17 (0.09-026) | 0.20 (0.12-0.27) | 0.19 (0.12-0.29) | 0.12-0.32 |
| ASAT (U/L) | 12 (6-23) | 12 (8-16) | 12 (4-17) | 10-30 |
| ALAT (U/L) | 10 (8-24) | 10 (4-18) | 8 (5-16) | 10-30 |
| LD (U/L) | 172 (141-268) | 178 (133-216) | 185 (134-222) | 150-250 |
| T4 (nmol/L) | 95 (75-130) | 110 (88-130) | 110 (97-150) | 58-148 |

## 2.4 SEMEN CHARACTERISTICS

In all partners of the women with irregular menstrual cycles and the women of the group with unexplained infertility a semen analysis (SA) was carried out additionally to the SAs that were performed during the fertility evaluation before admission to this study. A SA was performed in nine partners of the control group of assumed fertile women. Seven men of the last group had proven their fertility. The results of the SAs of each group are shown in table II.4.

**Table II.4** Semen parameters of the investigated groups Median (range)

| Semen Parameter | Control Group n=9 | Irregular Cycles n=15 | Unexplained Infertility n=20 |
|---|---|---|---|
| Volume (mL) | 5.2 (1.1-8.2) | 2.7 (1.1-10.0) | 3.9 (1.6-6.5) |
| Density (x$10^6$/mL) | 61 (20-330) | 45 (6-100) | 50 (8-190) |
| Motility (%) | 60 (30-100) | 50 (10-100) | 55 (5-100) |
| Motility grade | 4-5 (4-6) | 4-5 (2-6) | 4-5 (3-5) |
| Abnormal forms (%) | 35 (24-58) | 41 (27-65) | 36 (25-51) |

The criteria of this laboratory for a normal SA are: sperm density ≥20 x $10^6$/mL, percentage motility ≥50%, motility grade ≥4 and ≤40% abnormal forms.

In the control group five out of nine men had a normal SA. Three men had one abnormal semen parameter and one male had two abnormal semen characteristics. The latter male had proven his fertility.

Five partners of women with irregular menstrual cycles had a normal spermiogram. The remaining ten men of this group had one or two abnormal sperm characteristics. Despite the selection criterion of two normal SAs, only ten out of twenty males of the group with unexplained infertility had a completely normal SA. Six men had one slightly abnormal semen parameter and four males had a combination of two or three abnormal characteristics.

Furthermore the zona-free hamster ovum sperm penetration assay (SPA) was carried out in eight men of the control group, in fourteen partners of women with irregular cycles and in all men of the group with unexplained infertility. Prior to the incubation of the washed spermatozoa with the hamster oocytes the motility grade was determined.[4] The results of the SPA with regard to the motility after preincubation are shown in table II.5. None of the men with insufficient motility after preincubation had a positive SPA. In case of good motility after preincubation the majority of males in all three groups had a positive SPA. Despite proven fertility only four out of eight men showed a positive result in the SPA. The remaining three men had an insufficient motility after preincubation and one man had penetration rate of 0% in the SPA, although the motility after preincubation was sufficient. It was demonstrated in all three groups that in almost all semen samples which showed insufficient motility after preincubation and/or a nega-

tive SPA also one or more abnormal semen parameters were determined in the routine SA of the same semen sample. The majority of men with a normal spermiogram also had a positive SPA.

**Table II.5** Number of men of the investigated groups with a positive SPA (penetration rate >0%), out of subgroups with either sufficient, insufficient or without motility after preincubation

| | Control Group n=8 | Irregular Cycles n=14 | Unexplained Infertility n=20 |
|---|---|---|---|
| Motility grade after preincubation ≥4 | 4/5 | 7/9 | 14/15 |
| Motility grade after preincubation <4 | 0/1 | 0/3 | 0/3 |
| Immotile after preincubation, SPA cancelled | 2 | 2 | 2 |

## 2.5 SCHEDULE OF OBSERVATIONS

The aim of this study was to observe the hormonal pattern in normal and 'infertile' menstrual cycles. Frequent venous blood samples were taken during one menstrual cycle without medication. Blood samples were collected every other day during the follicular and luteal phase, and during the periovulatory phase blood samples were obtained daily. Saliva was obtained on two occasions during each phase of the cycle. Whole saliva was collected at home by the women and immediately frozen after collection. Blood and saliva were collected between 8 and 12 a.m., but not always at the same time.

To determine the phase of the cycle follicular growth and ovulation were observed by ultrasound (Real-time B scan, Toshiba Sonolayer-L SAL 20A, 2.4 MHz transducer). Depending on the duration of the previous cycles, the first ultrasound examination took place at around five days before the expected day of ovulation and examinations were continued until ultrasonographical signs of follicle rupture had occurred. Furthermore all women recorded a BBT chart.

Prophylactic ferrosulphate medication (Fero-Gradumet®, Abbott Nederland B.V.) was offered to all women during the study cycle.

## 2.6 IMMUNOCHEMICAL PROCEDURES IN SERUM AND SALIVA

### 2.6.1 Preparation of serum and saliva

Serum was obtained by vena puncture and after clotting and centrifugation, serum was frozen at -20° C until assays were performed. Saliva was collected at home and frozen until assayed.

The concentrations of thirteen different hormones and binding proteins involved in fertility regulation have been determined in the serum samples and eight of these were also quantified in the majority of the saliva samples collected.

In serum this series concerned: Luteinising hormone (LH), Follicle stimulating hormone (FSH), prolactin (PRL), and thyroxine ($T_4$), as well as the steroid hormones 17ß-oestradiol ($Oe_2$), progesterone (Prog), 17α-OH-progesterone (17OHP), androstenedione (Andr), testosterone (Test), Dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulphate (DHEAS) and cortisol, and sex-hormone binding globulin (SHBG). All the above mentioned steroid hormones have also been determined in the saliva. Some components allowed the direct determination in serum (LH, FSH, PRL, cortisol, DHEAS and SHBG) or saliva (DHEAS and cortisol), whereas the remaining components required a sample purification procedure prior to estimation. In the case of serum the quantitation of $Oe_2$, Prog, Test and DHEA was preceded by extraction of the samples with diethylether, whereas in the case of 17OHP and Andr the extraction was followed by chromatographic isolation of the steroids by applying Sephadex LH-20 prior to assay.

The determination of all steroid hormones in saliva (except DHEAS and cortisol) was performed after both diethylether extraction and Sephadex LH-20 separation.

All the applied organic solvents were of analytical grade and purchased from E. Merck, Darmstadt, F.R.G.

#### *2.6.1.1 Extraction with diethylether*

In order to allow the correction for procedural losses due to the sample purification procedures previous to immunochemical assay amounts of 1000 cpm of the appropriate tritiated steroid were added to 0.5 mL of serum or 4.0 mL of saliva samples and this was followed by incubation for 10 min at room temperature. Next, the serum and saliva samples were extracted twice with 4 mL and 16 mL diethylether, respectively, and the combined ether layers were dried under a stream of nitrogen. If the measurement of a given component only required extraction, the residues of the organic layers were dissolved in 0.5 mL assay buffer and then assayed, else residues of the the extracts were dissolved in 0.2 mL Iso-octane: Toluene: Methanol (I:T:M) = 90:5:5 (v/v) in order to prepare for the chromatographic separation procedure on micro columns filled with Sephadex LH-20. The percentages of recovered tritiated steroids after extraction were always >85%.

*2.6.1.2 Chromatography on Sephadex LH-20*

The chromatographic separation of the steroids from the diethyl-ether extracts into distinct effluents was performed on micro columns filled with 0.5 g Sephadex LH-20 (Pharmacia AB, Uppsala, Sweden). The columns were developed with the solvent mixture I:T:M = 90:5:5 and the locations of the different steroids in terms of effluent portions were: Prog 1.5-4.0 mL; Andr 3.5-7.0 mL; DHEA 6.0-9.0 mL; Test 8.5-12.5 mL; and 17OHP 10.5-14.0 mL. After this procedure $Oe_2$ was isolated at 2.0-6.0 mL of effluent by applying I:T:M = 60:20:20.

As can be derived from the locations of the respective steroids it is not possible to separate all the steroids in one distinct chromatography run. Thus, two different runs were performed to achieve the complete separation of all the steroids. The combinations Prog-DHEA-17OHP and Andr-Test-$Oe_2$ were selected and in practice, $^3$H-steroids added to serum or saliva (prior to extraction and subsequent chromatography) were recovered with percentages ranging between 65-85%.

Once the Sephadex LH-20 separation had been performed the effluents were dried under a stream of nitrogen and the residues were taken up in 0.5 mL of assay buffer. Figure 2.1 depicts the chromatograms of the two runs for the isolation of these steroids.

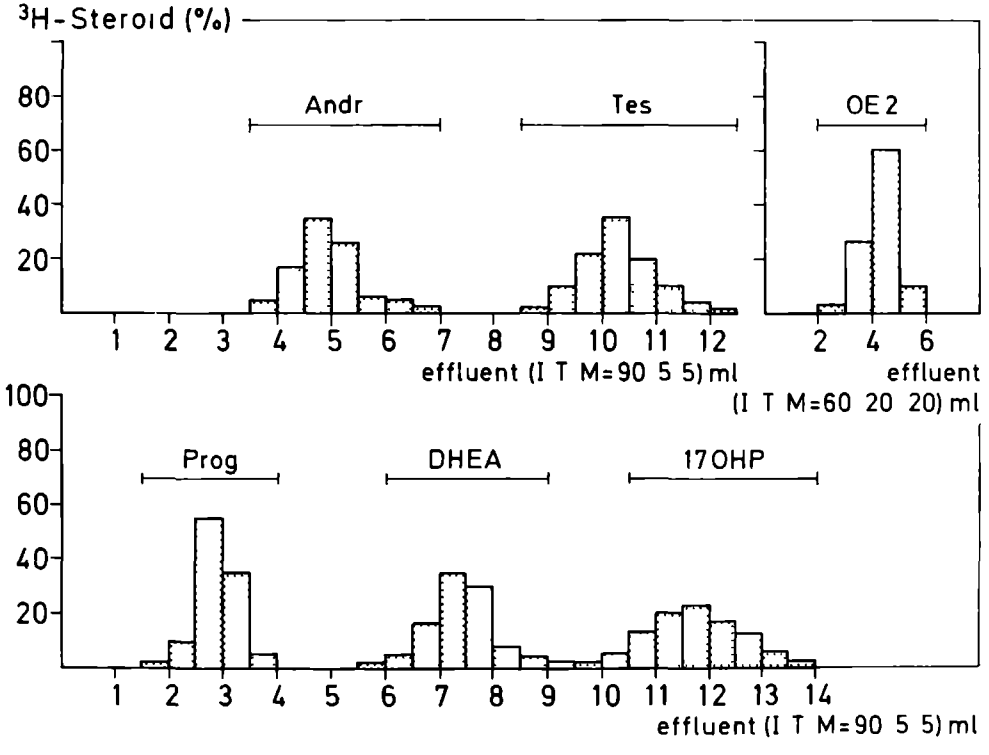


**Figure 2.1** Sephadex LH20 chromatograms for the isolation of steroids from saliva

### 2.6.2 Assay methods

Quantitation of the analytes contained in the serum or saliva samples (irrespective of whether direct assays or purification procedures prior to assay were involved) was performed with two types of immunoassays; competitive radioimmunoassays (RIA) on the one hand, or immunoradiometric "sandwich-type" assays (IRMA) on the other. In the case of each individual assay a series of known amounts of the respective standard was assayed to compose a stan-dard dose-response curve. From such curves the individual responses of the unknowns were read to calculate the concentrations of the analyte contained in these samples. In the case of RIA the response variable (i.e. the amount of radioactivity of a labelled standard preparation bound to the antibody) decreases with increasing amounts of standard whereas in the case of IRMA increas-ing amounts of radioactivity are observed with increasing amounts of standard.

#### *2.6.2.1 Radioactivity measurements and calculation procedures*

All the performed assays made use of radioactive labelled hormones or antibodies. For this purpose the majority of steroids involved (except for cortisol) applied tritium ($^{3}H$, a weak beta-emitting radionuclide) which substitutes up to six hydrogen atoms of the original steroid molecule. These commercially available steroid preparations (Amersham International plc, Amersham, England) were quantified by means of liquid scintillation counting for which we used a "1209 Rackbeta Primo" liquid scintillation counter (Pharmacia-Wallac Oy, Turku, Finland).

The other assays were based on the application of $^{125}I$ which is a gamma-emitting isotope. This radionuclide was coupled either tostandard material of the analyte to be measured or to one of the antibodies used. The quantitation of $^{125}I$ was detected directly in a "1261 Multigamma Counter" (Pharmacia-Wallac Oy, Turku, Finland).

Concentrations of the various analytes were calculated from the counting data using a sophisticated software package (RiaCalc LM Programme) commercially available from Pharmacia-Wallac Oy, Turku, Finland. In the case of scintillation counting the counting data were stored on a floppy diskette using a "Mariachi Seed 2" diskdrive (Mariachi Oy, Turku, Finland) and the calculations were performed afterwards. If on the other hand $^{125}I$ was involved the calculations were performed on-line with the RiaCalc package loaded into a PC while using the gamma counter.

Within the Riacalc programme RIA results were calculated follow-ing logit-log transformation of the dose-response data[5], whereas IRMAs were always based on calculations using "spline" functions.[6]

*2.6.2.2 Assay validation: reliability criteria*

The reliability of all the applied RIA and IRMA assays was investigated according to the following criteria:

- The analytical sensitivity of an assay in terms of the minimal detectable dose of analyte was defined in the case of RIA as the dose reading at zero-dose binding minus twice the standard error (SE). In the case of IRMA the dose reading at zero-binding plus twice the SE was used. Results of analytical sensitivities with each assay are given in Table II.6a.
- The precision of an assay was calculated from the means of duplicate measurements of several different serum and saliva pools, each after N consecutive assay runs.[5] In the case of the steroids, two different sample aliquots taken from one pool were assayed to cover the response range. Tables II.6b and II.6c depict the percent coefficients of variation (CV) of both within ($CV_w$) and between ($CV_b$) assay variabilities.
- The analytical specificity of the applied assays in terms of percent cross-reaction of potentially interfering peptide or steroid hormones has been tested according to the procedure described by Abraham.[7] Thus, the percent ratio of analyte standard over the amounts of potentially cross reacting components were calculated at response levels of $B/B_0 = 0.5$ and are given in the descriptions of the individual assays.
- The accuracy of the applied assays can be tested in two different manners: a) parallelism between linearised dose-response lines of standards and serially diluted unknowns ("parallel line assays", see Tables II.7a and II.7b) by calculating the percent recoveries of known amounts of analyte added to serum samples previous to extraction and chromatography covering the entire dose-response range. The results of these experiments are depicted in Table II.7b.

**Table II.6a** Sensitivity of thirteen assays

| Assay | Sensitivity | | Assay | Sensitivity | |
|---|---|---|---|---|---|
| LH | 1.5 | IU/L | $Oe_2$ | 5.0 | fmol/tube |
| FSH | 0.35 | IU/L | Prog | 25 | fmol/tube |
| PRL | 30 | mIU/L | 17OHP | 10 | fmol/tube |
| Cortisol | 10 | nmol/L | Andr | 5.0 | fmol/tube |
| SHBG | 1.0 | nmol/L | Test | 5.0 | fmol/tube |
| $T_4$ | 6.4 | nmol/L | DHEA | 7.5 | fmol/tube |
| | | | DHEAS | 7.5 | fmol/tube |

**Table II.6b** Precision of thirteen assays in serum

| Assay | Dimension | Pool Mean | No of Runs | Precision (%) CVw | CVb |
|---|---|---|---|---|---|
| LH | IU/L | 16 | 99 | 11 | 18 |
| | IU/L | 64 | 97 | 8.3 | 13 |
| FSH | IU/L | 5.1 | 65 | 13 | 16 |
| | IU/L | 17 | 62 | 8.1 | 12 |
| PRL | mIU/L | 430 | 88 | 5.8 | 10 |
| | mIU/L | 1200 | 88 | 4.9 | 8.5 |
| $Oe_2$ | pmol/L | 820 | 78 | 4.3 | 7.9 |
| Prog | nmol/L | 39 | 36 | 4.1 | 9.1 |
| 17OHP | nmol/L | 6.4 | 20 | 4.5 | 7.7 |
| Andr | nmol/L | 4.8 | 40 | 5.5 | 10 |
| Test | nmol/L | 2.6 | 123 | 4.9 | 6.6 |
| DHEA | nmol/L | 17 | 26 | 4.7 | 7.5 |
| DHEAS | µmol/L | 7.9 | 21 | 3.8 | 7.3 |
| Cortisol | µmol/L | 0.38 | 92 | 3.2 | 6.3 |
| SHBG | nmol/L | 9.6 | 20 | 3.7 | 7.7 |
| | nmol/L | 55 | 20 | 2.1 | 5.6 |
| | nmol/L | 110 | 20 | 2.8 | 4.8 |
| $T_4$ | nmol/L | 26 | 38 | 7.5 | 12 |
| | nmol/L | 99 | 42 | 4.7 | 7.3 |
| | nmol/L | 184 | 43 | 3.6 | 6.7 |

**Table II.6c** Precision of eight steroid assays in saliva

| Assay | Dimension | Pool Mean | No of Runs | Precision (%) CVw | CVb |
|---|---|---|---|---|---|
| $Oe_2$ | pmol/L | 67 | 11 | 4.0 | 6.6 |
| Prog | pmol/L | 240 | 10 | 10 | 11 |
| 17OHP | pmol/L | 120 | 9 | 6.8 | 11 |
| Andr | pmol/L | 310 | 9 | 6.7 | 12 |
| Test | pmol/L | 60 | 11 | 8.5 | 11 |
| DHEA | pmol/L | 310 | 11 | 5.9 | 8.4 |
| DHEAS | nmol/L | 6.6 | 10 | 4.2 | 5.2 |
| Cortisol | nmol/L | 15 | 15 | 6.1 | 12 |

### 2.6.3 Applied Assays

*2.6.3.1 Luteinising Hormone*

Serum concentrations of LH were measured by a specific homologous double antibody solid-phase RIA as described previously by Van Geelen et al.[8] The standard-doses in terms of 1st IRP 68/40 ranged from 4.0-250 IU/L. HCG cross-reacted for 100% whereas

the cross-reactivity of all other peptide hormones tested was negligible. Data on sensitivity and precision are given in Tables II.6a and II.6b. The data on accuracy are given in Table II.7a.

*2.6.3.2 Follicle Stimulating Hormone*

Serum concentrations of FSH were measured by a specific homologous double antibody solid-phase RIA described by Van Geelen et al.[8] Standard-doses in terms of 1st IRP 78/549 ranged from 1.6-100 IU/L and no cross-reactivity was observed with all peptide hormones tested. Sensitivity and precision figures are depicted in Tables II.6a and II.6b. The data on accuracy are given in Table II.7a.

*2.6.3.3 Prolactin*

Serum concentrations of PRL were measured by a competitive solid-phase RIA commercially available from Farmos Diagnostica (Turku, Finland). Standard-doses in terms of 1st IRP 75/504 ranged from 50-5000 mIU/L and cross-reactivity was <0.01% for all peptide hormones tested including hGH and hPL. Assay sensitivity and precision data are shown in Tables II.6a and II.6b. The data on accuracy are given in Tables II.7a and II.7b.

*2.6.3.4 17β Oestradiol*

The levels of $OE_2$ in serum (pmol/L) and saliva (pmol/L) were measured with the specific charcoal RIA as described by Thomas et al.[9] This assay was preceded by the sample purification procedure as indicated above and made use of an antiserum raised in rabbits against $OE_2$-6-(O-carboxymethyl)oxime:BSA. Standard-doses in terms of $OE_2$ ranged from 10-1500 fmol/tube. The $OE_2$ assay cross-reacted only with $OE_1$ for 1.0%, whereas all other steroids tested showed cross-reactions of <1%. Sensitivity and precision data for serum and saliva are given in Tables II.6a, II.6b and II.6c. Data on accuracy are depicted in Tables II.7a and II.7b.

*2.6.3.5 Progesterone*

Serum and salivary Prog measurements were performed by a specific charcoal RIA according to Thomas et al.[9] using an antibody raised in rabbits against 11α-hydroxy-Prog-hemisuccinate: BSA. Standard-doses ranged from 50-3200 fmol Prog/tube. Results in saliva are expressed in pmol/L and those in serum samples in nmol/L. The applied extraction and chromatographic purification procedures previous to assay have been described above. Except for 11-deoxy-corticosteron which has a cross-reaction of 3.8%, all steroids tested cross-reacted for <1%. Data on sensitivity and precision for both serum and saliva are shown in Tables II.6a, II.6b and II.6c, while the accuracy of the assay is given in Tables II.7a and II.7b.

*2.6.3.6 17α-hydroxyprogesterone*

The levels of 17OHP in both serum (nmol/L) and saliva (pmol/L) were determined with a highly specific charcoal RIA as described by Dony et al.[10] The assay was preceded by

the sample purification procedure already described above. The applied antiserum was purchased from Radioassay Systems Laboratories Inc. (Carson, CA, USA) and had been raised in rabbits against 17OHP-7-carboxyethylthioether:BSA.
Standard-doses in terms of 17OHP ranged from 25-3000 fmol/tube. Progesterone and 17α-hydroxypregnenolone showed 0.4% and 1.1% cross-reactivity whereas all other relevant steroids cross-reacted <0.01%. Sensitivity and precision data are given in Tables II.6a, II.6b and II.6c. The data on accuracy are given in Tables II.7a and II.7b.

*2.6.3.7 Androstenedione*
The concentrations of Androstenedione in serum (nmol/L) and saliva (pmol/L) were measured by a charcoal RIA following the extraction and chromatography procedures described above. The RIA applied a commercially available antiserum (Radioassay Systems Laboratories Inc., Carson, CA, USA) which had been raised in rabbits against Andr-19-carboxymethylether:BSA. Standard-doses ranged from 10-1600 fmol Androstenedione/tube. Relevant cross-reactions were observed with DHEA (4.9%), DHEAS (6.9%), and 5α-androstanedione (16.8%) whereas cross-reactivity of all other steroids tested was <0.5%. Assay sensitivity and precision for both serum and saliva measurements are shown in Tables II.6a to II.6c. The data on accuracy are given in Tables II.7a and II.7b.

*2.6.3.8 Testosterone*
Serum (nmol/L) and salivary (pmol/L) Testosterone concentrations were measured following sample purification (see above) by specific charcoal RIA as described by Dony et al.[10] The assay uses an antibody raised in rabbits against Tes-3-(O-carboxymethyl)oxime: BSA. Testosterone standards ranged from 10-1500 fmol/tube and the cross-reactivities for all steroids tested were <0.05% except for 5α-and 5ß dihydrotestosterone which showed cross-reactions of 48% and 14%, respectively. Sensitivity and precision data are shown in Tables II.6a to II.6c. The data on accuracy are given in Tables II.7a and II.7b.

*2.6.3.9 Dehydroepiandrosterone*
The concentrations of DHEA in serum (nmol/L) and in saliva (pmol/L) were measured following sample purification (see above) with a highly specific charcoal RIA as described by Reijnders.[11] The RIA used an antibody purchased from Radioassay Systems Laboratories (Carson, Ca, USA). It has been raised in rabbits against 15ß-carboxyethylmercapto-DHEA:BSA. Standard-doses ranged from 15-1800 fmol DHEA/tube whereas the cross-reactivity of all the relevant steroids tested was <0.3%. Data on sensitivity and precision for both serum and saliva are shown in Tables II.6a, II.6b and II.6c. The data on accuracy are given in Tables II.7a and II.7b.

*2.6.3.10 Dehydroepiandrosterone-sulphate*
The concentrations of DHEAS in serum (µmol/L) and saliva (nmol/L) without were measured without any pre-treatment while using a specific charcoal-based RIA

The concentrations of DHEAS in serum (µmol/L) and saliva (nmol/L) without were measured without any pre-treatment while using a specific charcoal-based RIA described by Reijnders.[11] This assay used an antibody raised in sheep against DHEA-3ß-monohemi-succinate:HSA (Radioassay Systems Laboratories, Carson, CA, USA). The DHEAS standards ranged from 15-2000 fmol/tube. Cross-reactions for all steroids tested were <0.27% with the exception of DHEA which showed a cross-reaction of 73%. The high percentage of cross-reactivity of DHEA did not affect the DHEAS levels since the concentration-ratio of DHEA/DHEAS in serum is <0.001 and with saliva <0.01. Data on assay sensitivity and precision are depicted in Tables II.6a to II.6c. The data on accuracy are given in Tables II.7a and II.7b.

*2.6.3.11 Cortisol*

Measurements of serum (µmol/L) and salivary (nmol/L) cortisol levels were performed with a commercially available competitive coated tube RIA (Baxter Travenol Clinical Assays Inc., Boston, Mass, USA) as described previously by Dony et al.[10] Standard-doses ranged from 30-1700 nmol Cor/L. Cross-reactivity was measured for prednisolone (73%) and 6-methyl-prednisolone (18%) whereas the cross-reaction of other relevant steroid hormones tested was almost negligible.

Since salivary cortisol levels were relatively low a small modification to the kit procedure was introduced by changing the sample volume from 0.01 mL to 0.10 mL without any effect on the assay results. Assay sensitivity and precision data of serum and saliva measurements are given in Tables II.6a to II.6c and accuracy is documented in Tables II.7a and II.7b.

*2.6.3.12 Sex Hormone Binding Globulin*

Serum concentrations of SHBG were measured by a commercially available (Farmos Diagnostica, Turku, Finland) non-competitive liquid-phase IRMA according to the method of Hammond et al.[12] Standards were used in the range 6.25-200 nmol SHBG/L while no human serum proteins are known to cross-react with this assay. Data on precision and sensitivity are shown in Table II.6a and II.6b. The data on accuracy are given in Table II.7b.

*2.6.3.13 Thyroxin*

Measurements of serum $T_4$ levels were performed with a commercially available competitive coated RIA (Baxter Travenol Clinical Assays Inc., Boston, Mass., U.S.A.) as applied previously by Reijnders.[11] Standard-doses ranged from 13-260 nmol $T_4$/L. Cross-reactivity was determined for L-$T_3$ (2.6%) and D-$T_3$ (2.1%) and cross-reactions.

**Table II.7a** Accuracy: Parallel line assay in serum

| Assay | Dimension | $B/B_0$- Range(%) | N | Dose Mean ± SE | (%CV) |
|---|---|---|---|---|---|
| LH | IU/L | 89 - 20 | 12 | 91.1 ± 7.9 | (8.6) |
| FSH | IU/L | 79 - 11 | 17 | 76.6 ± 8.6 | (11.2) |
| PRL | mIU/L | 88 - 28 | 10 | 2330 ± 360 | (15.2) |
| $Oe_2$ | pmol/L | 89 - 32 | 10 | 1270 ± 117 | (9.2) |
| Prog | nmol/L | 83 - 16 | 8 | 24.8 ± 2.6 | (10.3) |
| 17OHP | nmol/L | 85 - 20 | 9 | 15.9 ± 1.5 | (9.3) |
| Andr | nmol/L | 87 - 25 | 10 | 6.1 ± 0.14 | (6.8) |
| Test | nmol/L | 80 - 16 | 10 | 13.4 ± 1.1 | (8.2) |
| DHEA | nmol/L | 81 - 10 | 12 | 33.5 ± 3.8 | (11.3) |
| DHEAS | µmol/L | 79 - 17 | 8 | 9.1 ± 0.70 | (7.8) |
| Cortisol | µmol/L | 81 - 25 | 9 | 0.40 ± 0.03 | (6.4) |

**Table II.7b** Accuracy: Recovery of standard analyte added to serum

| Assay | N | Recovery (%) Mean ± SE | (%CV) |
|---|---|---|---|
| PRL | 10 | 103 ± 22 | (21) |
| $Oe_2$ | 30 | 100 ± 10 | (10) |
| Prog | 16 | 97 ± 15 | (15) |
| 17OHP | 24 | 96 ± 11 | (11) |
| Andr | 28 | 97 ± 13 | (13) |
| Test | 18 | 99 ± 7.8 | (7.9) |
| DHEA | 16 | 101 ± 12 | (11) |
| DHEAS | 20 | 97 ± 5.9 | (6.1) |
| Cortisol | 20 | 102 ± 4.0 | (3.9) |
| SHBG | 10 | 91 ± 5.5 | (6.0) |
| $T_4$ | 20 | 100 ± 6.3 | (6.3) |

## 2.7 DATA HANDLING AND STATISTICAL ANALYSIS

### 2.7.1 Data handling

The intention of the study was to obtain blood samples every two days during the follicular and luteal phase and daily during the peri-ovulatory phase. Furthermore saliva was designed to be collected on two occasions during each phase of the cycle. Retrospectively in some women blood and/or saliva samples were incomplete during one or more phases of their cycle.

The day of the highest LH concentration is designated day 0. The day of the LH peak was obvious in 44 out of the 55 women. The remaining eleven women showed no clear LH peak because of incomplete daily serum sampling during the periovulatory period.

To determine the expected day of the LH peak the available hormonal data of these women were submitted to five independent observers who are specialised in infertility problems. The day which was designated day 0 by a majority of the observers was assumed to be the day of the LH peak.

The days preceding the mid-cycle LH peak are called day -1, -2, -3 etc. and the days following the day of the LH peak are called +1, +2, +3 etc. The follicular phase is defined as the period from the first day of menstruation to day -3. The periovulatory phase include days -2 to +1 and the luteal phase is defined as the period from day +2 until the day before the next menstruation. The time sequenced data of a hormone throughout the menstrual cycle is called a 'response curve'.

Because of the schedule of blood sampling (i.e. every other day) it can be expected that during the follicular and luteal phase half of the samples have been collected on the even days and the other half of the samples on the odd days. If one would connect the calculated mean values of a given hormonal parameter of every sampling day of the cycle, an artificial saw-edged curve might originate from this, as a result of the random division of the women into the 'even day' and 'odd day' groups. To avoid this artificial saw-edged patterns of hormonal parameters in serum, two successive days have been taken together during the follicular and luteal phase. In case of two serum samples taken incidently on both days, only the hormonal results of the second sample was disgarded. Thus all data for a given day are equally weighed in the analysis.Table II.8 gives a survey of the number of patients with a serum sample at the indicated day of the cycle and the chosen grouping of the samples. This table also shows the collected number of saliva samples on each day of the cycle. In the follicular phase the median value of the saliva measurements of day -12 to -3 have been used for statistical analysis. In the periovulatory phase the first or only values on days -2 and -1, and on days 0 and +1,

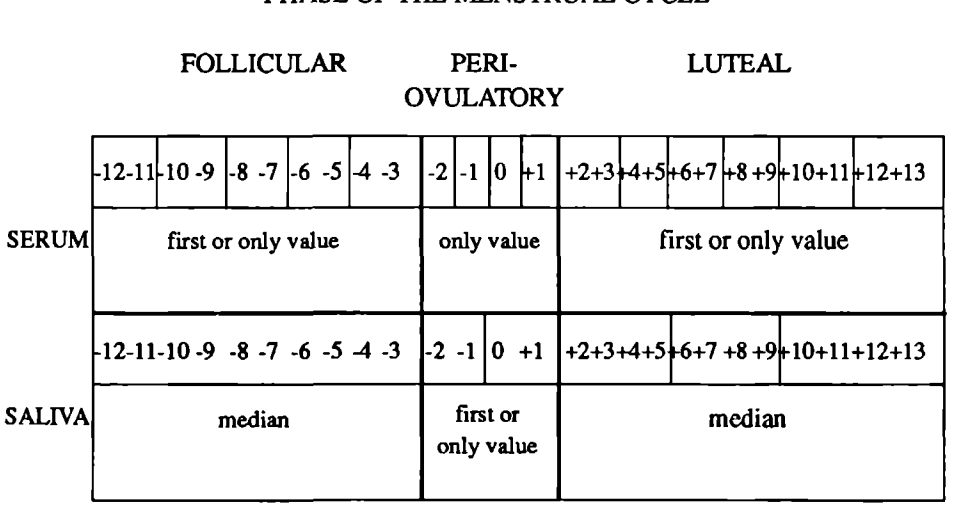


**Figure 2.2** Grouping of the hormonal data of serum and saliva for statistical analysis

respectively, have been used. In the luteal phase the median values of the periods +2 to +5, +6 to +9 and +10 to +13 have been used. Figure 2.2 summarises the grouping of the hormonal data of saliva and serum for statistical analysis.
In the figures of the hormonal parameters as shown in chapter 3 and 4 the mean ± the standard error of the mean (SEM) of each hormonal parameter is plotted against the moment of the menstrual cycle, whereby the cycle is divided into periods in the way described above i.e. -12/-11; -10/-9; ..... -4/-3; -2; -1; 0; +1; +2/+3; +4/+5 .....+12/+13.

### 2.7.2 Statistical analysis

To compare the hormonal patterns (=response curves) of each of the two infertile groups with the control group, the distribution-free statistical test described by Koziol et al.[13] has been used. This test is suitable in the comparison between response curves of two or more groups when incomplete observations are present and where parametric models for hormonal response curves are not available. This test has been performed separately for each of the three phases of the investigated menstrual cycle (follicular, peri-ovulatory and luteal).

In case the distribution of the hormonal parameter is not dependent on the day of the cycle the differences between the values of the infertile and control group have been analysed with the Wilcoxon (two sample) test based on the median value for each patient.

The Wilcoxon test proceeds as follows: The observations, the concentrations of a hormone of a defined moment of the cycle, are ranked from low to high. Each patient of the total study population is given a rank, from 1 to 55. For each group the mean rank is calculated. The difference between two mean ranks is the Wilcoxon coefficient (un-paired case). Group 1 is higher than group 2 means that the mean rank of group 1 is higher than the mean rank of group 2. In case of more observations during the cycle (response curve) for each group the sum of the mean ranks is calculated. Group 1 is higher than group 2 means that the sum of the mean ranks of group 1 is higher than the sum of group 2. The test is now called the test of Koziol and the sum of the ranks refer to an appropriate table of critical levels. This test is sensitive for differences between groups, which are in the same direction, i.e. always higher during the menstrual cycle.

The level of significance selected is 5 percent.

**Table II.8** Survey of the grouping of the serum and saliva samples of one menstrual cycle for the three investigated groups. The first column of each group shows the collected number of samples for each day of the cycle. The second column of each group gives, for the defined periods of the cycle, the number of samples used for analysis.

| | SERUM | | | | | | SALIVA | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cycle day | Control Group n=20 | | Irregular Cycles n=15 | | Unexplained Infertility n=20 | | Control Group n=20 | | Irregular Cycles n=15 | | Unexplained Infertility n=20 | |
| -12 | 7 | 11 | 4 | 9 | 4 | 10 | 2 | 20 | 3 | 13 | 0 | 13 |
| -11 | 4 | 5 | 7 | | | | 1 | | 0 | | 2 | |
| -10 | 10 | 19 | 7 | 14 | 11 | 18 | 6 | | 2 | | 1 | |
| -9 | 9 | | 7 | | 10 | | 2 | | 4 | | 1 | |
| -8 | 9 | 18 | 5 | 11 | 8 | 16 | 2 | | 4 | | 1 | |
| -7 | 13 | | 7 | | 8 | | 9 | | 3 | | 2 | |
| -6 | 11 | 19 | 8 | 12 | 11 | 17 | 7 | | 1 | | 4 | |
| -5 | 15 | | 7 | | 8 | | 3 | | 4 | | 6 | |
| -4 | 11 | 17 | 11 | 14 | 11 | 20 | 5 | | 5 | | 2 | |
| -3 | 15 | | 11 | | 14 | | 9 | | 2 | | 7 | |
| -2 | 15 | 15 | 12 | 12 | 14 | 14 | 7 | 14 | 8 | 10 | 10 | 16 |
| -1 | 17 | 17 | 12 | 12 | 17 | 17 | 8 | | 2 | | 7 | |
| 0 | 19 | 19 | 14 | 14 | 18 | 18 | 9 | 15 | 5 | 8 | 8 | 9 |
| +1 | 15 | 15 | 12 | 12 | 18 | 18 | 6 | | 3 | | 4 | |
| +2 | 18 | 18 | 6 | 12 | 15 | 19 | 6 | 16 | 5 | 10 | 4 | 18 |
| +3 | 9 | | 9 | | 16 | | 3 | | 3 | | 6 | |
| +4 | 12 | 18 | 6 | 14 | 9 | 18 | 8 | | 3 | | 7 | |
| +5 | 8 | | 8 | | 10 | | 2 | | 3 | | 7 | |
| +6 | 6 | 18 | 5 | 12 | 8 | 16 | 4 | 18 | 3 | 10 | 3 | 14 |
| +7 | 12 | | 7 | | 8 | | 6 | | 3 | | 4 | |
| +8 | 6 | 19 | 6 | 12 | 10 | 15 | 3 | | 2 | | 8 | |
| +9 | 14 | | 6 | | 5 | | 5 | | 2 | | 2 | |
| +10 | 4 | 13 | 6 | 13 | 13 | 19 | 3 | 13 | 3 | 9 | 4 | 15 |
| +11 | 9 | | 7 | | 7 | | 7 | | 4 | | 4 | |
| +12 | 7 | 13 | 7 | 11 | 9 | 17 | 4 | | 2 | | 4 | |
| +13 | 6 | | 4 | | 9 | | 1 | | 0 | | 3 | |

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# CHAPTER 3

# ENDOCRINOLOGICAL PARAMETERS OF THE GROUP WITH IRREGULAR CYCLES COMPARED TO THE CONTROL GROUP

## 3.1 INTRODUCTION

Ovarian dysfunction is a major cause of infertility. Approximately 30% to 40% of female infertility patients present with this problem. (see for review 1) An irregular cycle is not necessarily an anovulatory and/or an infertile cycle. Even though ovulation can occur in menstrual cycles of long duration, its relative infrequency decreases the chances for conception. Abnormal menstrual cycles can also have a negative effect on the target organs for sex steroids, e.g. the cervical mucus, and this may represent an indirect cause of infertility.

Deviations of the normal pattern of the menstrual cycle can be caused by various factors. Dysfunction can occur at hypothalamic, pituitary or ovarian level, it can be due to systemic diseases or thyroid or adrenal dysfunction.

A meaningful therapeutic approach to female infertility due to insufficient menstrual cycles is completely dependent on a adequate diagnostic evaluation. Both careful medical history and BBT records are helpful in assessing the pattern of the menstrual cycle. However, usually these methods neither give clues to the cause of an eventual abnormality, nor to the therapeutic approach for the infertility problem.

In order to evaluate the role of various endocrine parameters in the abnormal menstrual cycle and to see if hormonal determinations could reveal the cause of cycle disturbance, frequent sampling of serum was carried out in 15 women with abnormal menstrual patterns. During the same cycles saliva was also collected for determinations. The concentrations and cyclical patterns of the steroid levels in serum and saliva were compared. The results of the infertile women with irregular menstrual cycles were compared with data obtained from normal ovulating women, who were supposed to be fertile.

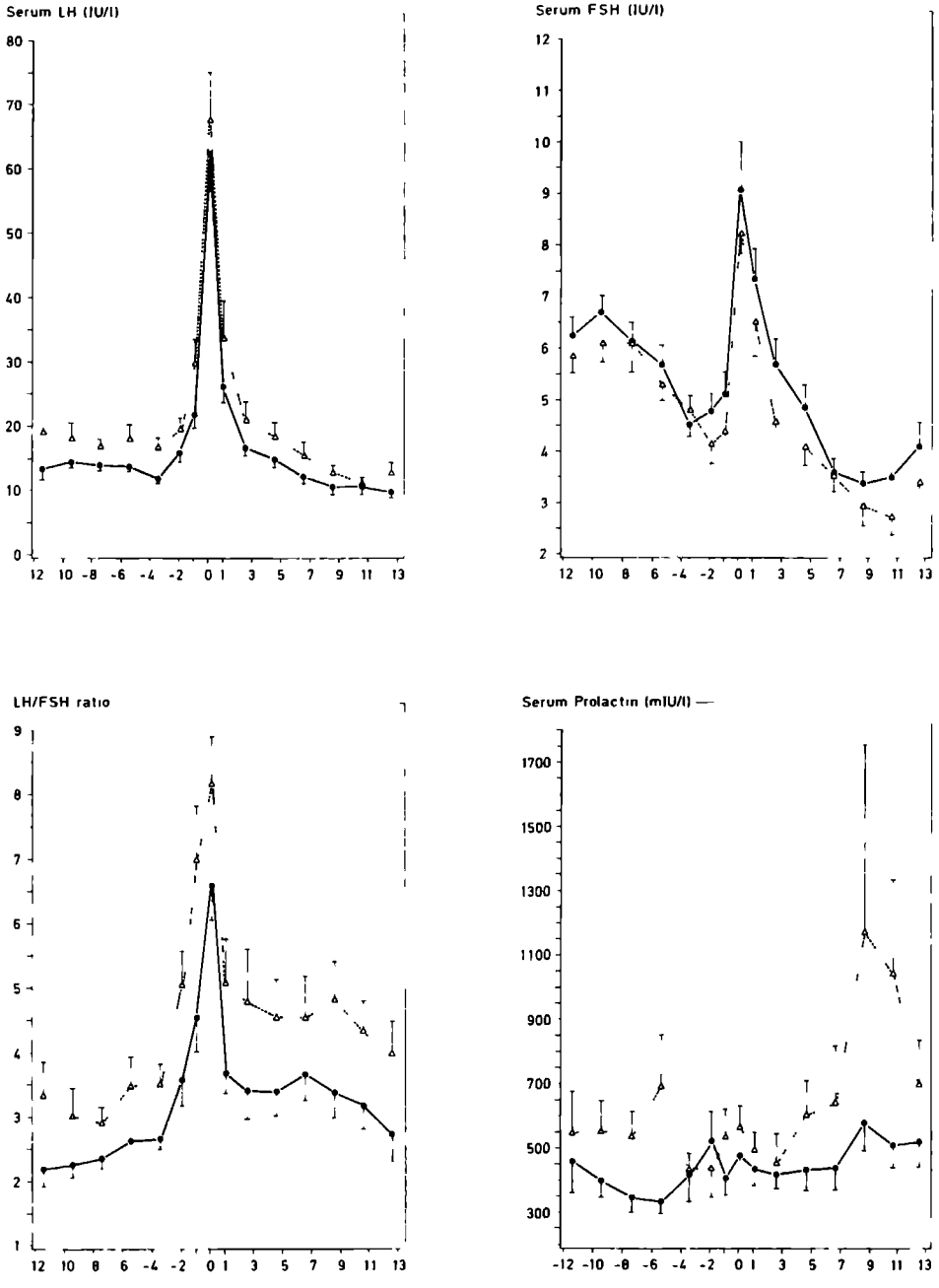


**Figure 3.1** Serum levels of LH, FSH, LH/FSH ratio and prolactin during one menstrual cycle of 15 women with irregular cycles (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

## 3.2 RESULTS

### 3.2.1 Endocrinological parameters in serum

*3.2.1.1 LH, FSH and the LH/FSH ratio (Fig 3.1)*

Figure 3.1 presents the patterns of LH, FSH and the LH/FSH ratio of the group with irregular cycles (dotted line) and the control group (drawn line). During the whole cycle the levels of LH tended to be higher in the women with irregular cycles in comparison to the control group, especially during the follicular (p= 0.06) and periovulatory phases (p=0.09).

The FSH levels of the women with irregular cycles were slightly lower than in the fertile women, but this difference never reached statistical significance.

As a result of the slightly increased LH- and low-normal FSH-concentrations, the LH/FSH ratio was elevated in the group with irregular cycles in comparison to the normal women. (follicular phase, p=0.02, periovulatory phase, p=0.05 and luteal phase, p= 0.09). For both groups the LH/FSH ratios were higher in the luteal phase compared to the follicular phase.

*3.2.1.2 Prolactin (Fig 3.1)*

During the follicular phase a tendency existed to higher prolactin levels in the women with irregular cycles (p=0.07). In the periovulatory and luteal phases no significant difference was found between the control group and the group with irregular cycles. Some women with irregular cycles showed elevated prolactin levels during the luteal phase. These findings are responsable for the wide range of prolactin concentrations during this period in this group which explains the lack of statistical difference between the two groups.

**Individual observations**

Eight women of the control group incidentally showed a prolactin concentration above 800mIU/L, the upper limit of normal in our laboratory, and three women had elevated prolactin levels on four, six and seven occasions, respectively, during the investigated cycle. None of the prolactin concentrations of the women of the control group exceeded 1500 mIU/L, and the length of their cycles was within the normal range. The mean concentration of prolactin of each woman during her studied cycle was under 800m IU/L except for one woman who had a mean prolactin level of 896 mIU/L.

In the group with irregular cycles, four women occasionally showed a prolactin concentration above 800 mIU/l and four women mostly had elevated prolactin levels throughout the observed menstrual cycle. The mean prolactin levels of the latter four women were 907, 1094, 1243 and 1435 mIU/L, respectively. They all received bromocriptine treatment for at least 6 months and three of these women conceived whilst on this treatment.

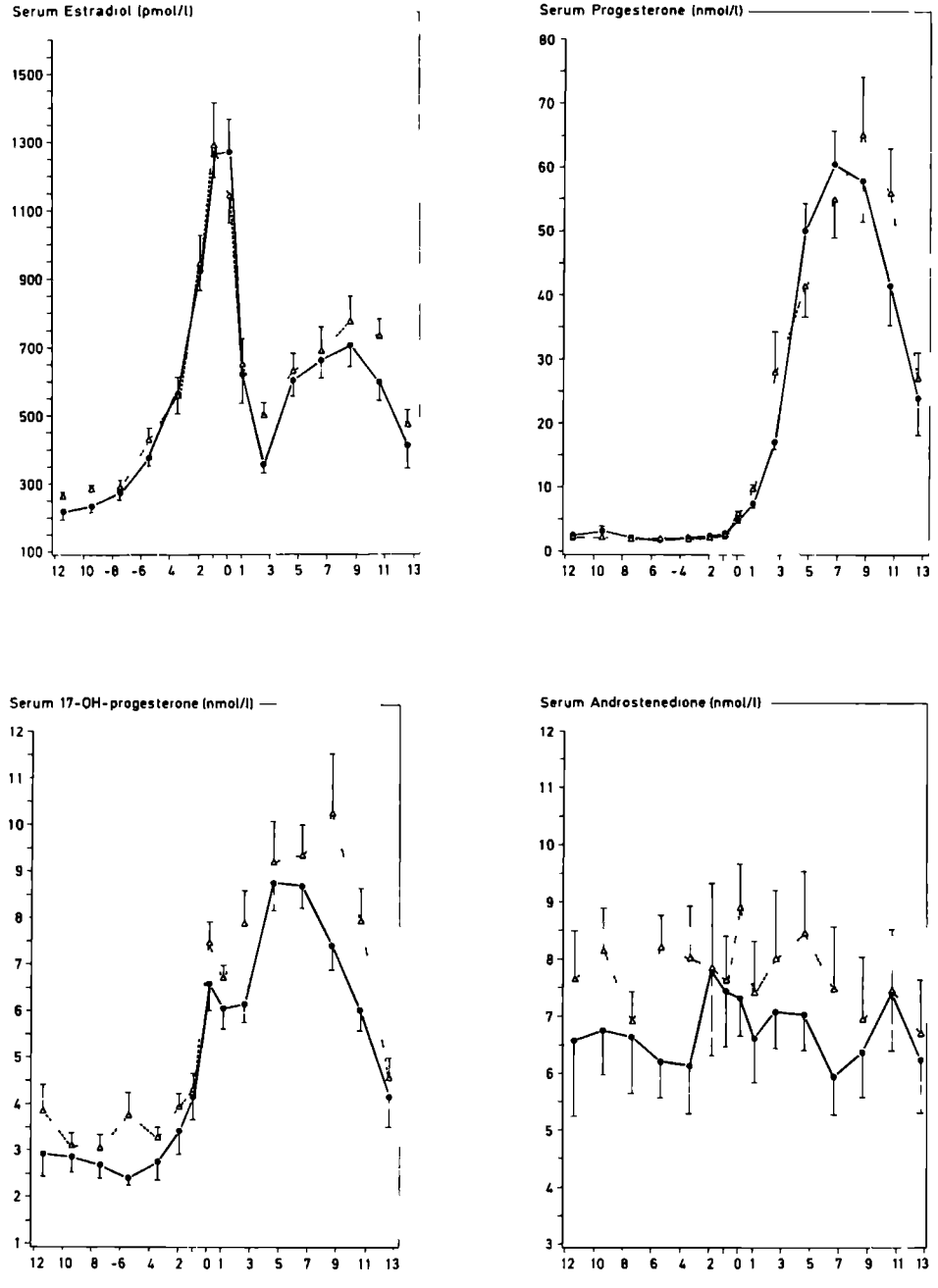


**Figure 3.2** Serum levels of 17ß-oestradiol, progesterone, 17α-OH-progesterone and androstenedione during one menstrual cycle of 15 women with irregular cycles (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

*3.2.1.3 17ß-Oestradiol (Fig 3.2)*

In both groups the midcycle LH surges were preceded by 17ß-oestradiol peaks. In most women the highest level of 17ß-oestradiol was found on cycle day -1 and in a minority the highest LH and 17ß-oestradiol concentration coincided on the same day. The maximum level of 17ß-oestradiol in the luteal phase was on day +8/+9 in both groups.

In each corresponding phase no statistical difference in 17ß-oestradiol levels was found between the group with irregular cycles and the control group.

*3.2.1.4 Progesterone (Fig 3.2)*

No significant differences in progesterone levels were found between the women with irregular cycles and the women with normal menstrual cycles. In both groups the first day of significant increase in progesterone concentration, i.e. an elevation of more than twice the inter-assay variability, was on cycle day 0 or on the day before, cycle day -1.

The maximal level of progesterone in the luteal phase was on day +8/+9 in the group with irregular cycles and on day +6/+7 in the control group.

*3.2.1.5 17α-OH-Progesterone (Fig 3.2)*

During the follicular phase of the cycle, the levels of 17α-OH-progesterone were almost constant and not different in both groups. During the periovulatory phase the 17α-OH-progesterone concentrations in the group with irregular cycles tended to be higher than in the control group ($p=0.07$).In both groups a significant increase of the 17α-OH-progesterone level was found on the day of the LH peak or on the day preceding the LH surge. In both groups a small 17α-OH-progesterone peak was seen coincident with the LH peak.

During the luteal phase a second rise appeared with maximal levels between days +4 and +9 in both groups.

*3.2.1.6 Androstenedione (Fig 3.2)*

The androstenedione concentrations of the women with irregular cycles tended to be higher in comparison to the concentrations of the women with normal cycles but this difference was not significant. Figure 3.2 shows no clear cyclical pattern, neither in the control group nor in the group with irregular cycles. However, considering the individual woman the highest concentration of androstenedione was mostly detected during the periovulatory phase of the cycle.

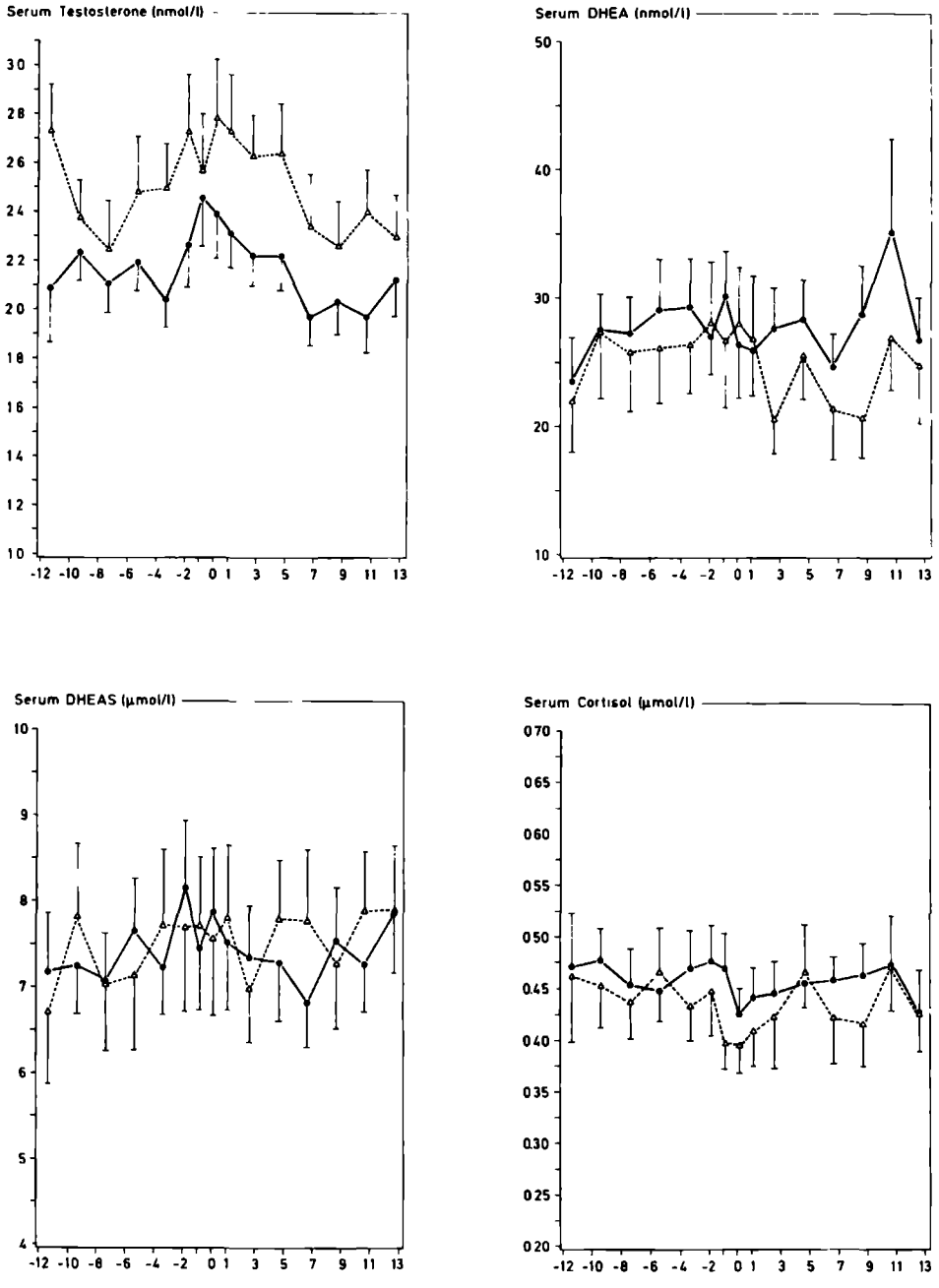


**Figure 3.3** Serum levels of testosterone, DHEA, DHEAS and cortisol during one menstrual cycle of 15 women with irregular cycles (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

*3.2.1.7 Testosterone (Fig 3.3)*

Throughout the observation period total testosterone concentrations were mostly higher in women with irregular cycles. (follicular phase p=0.05, periovulatory phase p=0.09 and luteal phase p=0.21) In the women with irregular cycles no obvious cyclical pattern of testosterone levels was seen: individual women demonstrated highest testosterone concentrations at different phases of their cycles. In the control group the highest testosterone concentration was mostly determined during the periovulatory phase and also, the lowest testosterone concentration was never seen during this period.

*3.2.1.8 DHEA, DHEAS (Fig 3.3)*

As for the DHEA and DHEAS concentrations, no difference between the two groups, and no cyclical pattern was found.

*3.2.1.9 Cortisol (Fig 3.3)*

The cortisol concentrations of both groups were not different. Neither in the women with irregular cycles, nor in the normal women a cyclical pattern of cortisol was seen.

*3.2.1.10 SHBG and Free Androgen Index*

In four women of the control group and in one woman with an irregular cycle SHBG was determined throughout the whole cycle. None of these five women showed a cyclical pattern for SHBG. In the remaining women of the control group and the group with irregular cycles SHBG was determined once in the very early follicular phase. The median concentration of early follicular SHBG in both groups was 50 nmol/L. The concentrations in the group with normal cycles ranged from 34 to 93 nmol/L and in the group with irregular cycles from 13 to 98 nmol/L. The free androgen index (FAI, i.e. the ratio of total serum testosterone to SHBG multiplied by 100) did also not differ between the two groups either and ranged from 1.51 to 10.3 in the control group and from 1.79 to 11.5 in the women with irregular cycles.

**Individual observations**

One woman in the control group had high serum testosterone concentrations (range 2.7 to 4.5 nmol/L) and also a high FAI (10.3), nevertheless she had normal menstrual patterns and no hyperandrogenic symptoms.

The woman with an irregular cycle and a FAI of 11.5 had normal serum testosterone levels (range 1.5 to 2.7, median 2.2 nmol/L). Her early follicular SHBG concentration was 13 nmol/L; her serum 17ß-oestradiol levels during the early follicular period were normal.

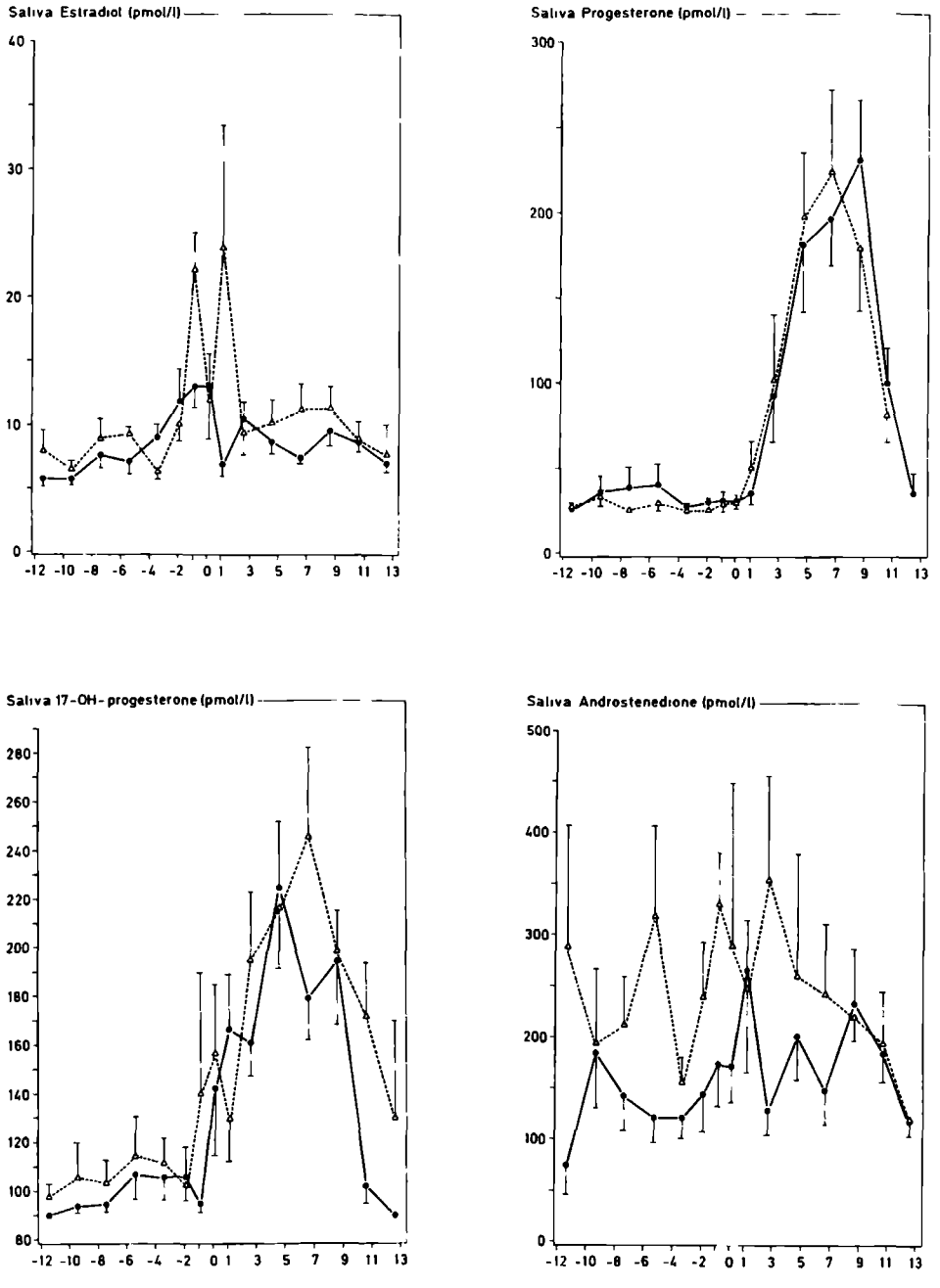


**Figure 3.4** Saliva levels of 17ß-oestradiol, progesterone, 17α-OH-progesterone and androstenedione during one menstrual cycle of 15 women with irregular cycles (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

### 3.2.2 ENDOCRINOLOGICAL PARAMETERS IN SALIVA

*3.2.2.1 17ß-Oestradiol (Fig 3.4)*

No significant difference was found in the concentrations of saliva 17ß-oestradiol between the control group and the group with abnormal cycles. Neither was there a difference in the ratio of saliva to serum oestradiol between the two groups.

Because of the limited number of saliva samples, no conclusions can be drawn on the cyclical pattern of 17ß-oestradiol in saliva. The saliva 17ß-oestradiol peaks on days -1 and +1 in the group with irregular cycles (see figure 3.4) are probably artefacts, caused by the low number of samples taken on the days mentioned above.

*3.2.2.2 Progesterone (Fig 3.4)*

A clear cyclical pattern was seen in saliva progesterone levels synchronously to that of the serum levels. The highest progesterone concentrations were seen between days +4 and +9. No difference in saliva progesterone levels were found between the two groups.

An increase in progesterone concentration just prior to ovulation could not be demonstrated in saliva.

*3.2.2.3 17α-OH-Progesterone (Fig 3.4)*

17α-OH-Progesterone concentrations in saliva were higher in the women with irregular cycles in comparison to the normal women. During the luteal phase this difference was statistically significant ($p=0.02$). In both groups a cyclical pattern could be recognised. A small peak during the periovulatory phase was seen in saliva as well as in serum.

*3.2.2.4 Androstenedione (Fig 3.4)*

The group with abnormal menstrual cycles had higher androstenedione levels in comparison to the control group ($p=0.08$). The highest saliva androstenedione concentration was mostly found during the periovulatory period.

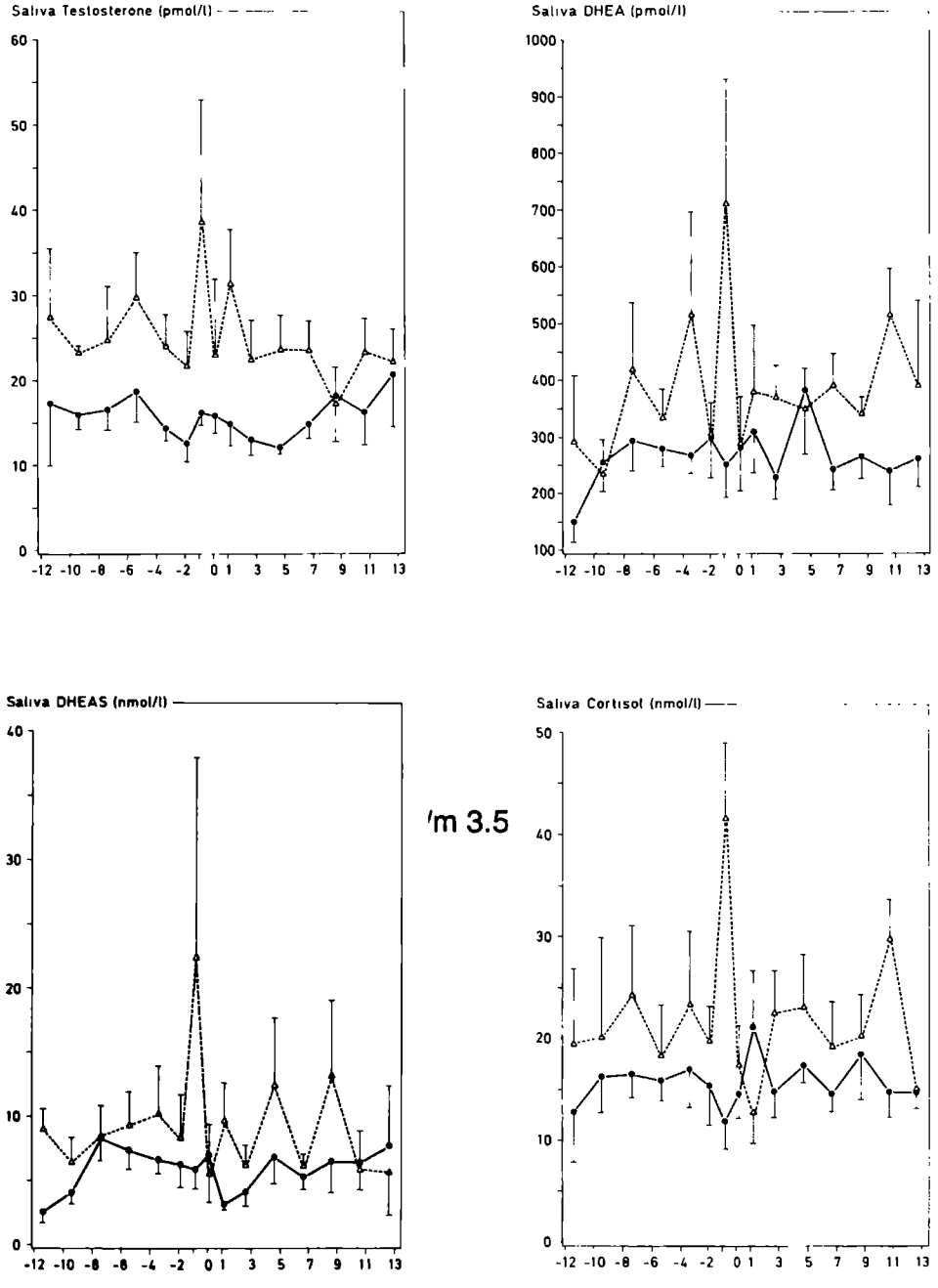


**Figure 3.5** Saliva levels of testosterone, DHEA, DHEAS and cortisol during one menstrual cycle of 15 women with irregular cycles (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)
Data were arranged according to the deviations of the LH peak (= day 0)

*3.2.2.5 Testosterone (Fig 3.5)*

The saliva concentrations of testosterone were markedly elevated in the women with irregular cycles in comparison to the control group (p=0.003).

Due to the limited number of observations, no conclusions could be drawn on the presence or absence of a cyclical pattern of testosterone in saliva.

*3.2.2.6 DHEA, DHEAS (Fig 3.5)*

The concentrations of both hormones, DHEA and DHEAS, were higher in the group with abnormal cycles compared to the control group (p= 0.04 and p=0.12, respectively), but no cyclical pattern was present.

*3.2.2.7 Cortisol (Fig 3.5)*

The saliva cortisol concentrations did not differ significantly between the two groups.

**Table III.1** P–values of the comparison of the collected hormonal data of the group with irregular cycles versus the control group. (Statistical test described by Koziol).
For comparison of 17–OHP, A–dione, testosterone, DHEA, DHEAS and cortisol in saliva the median value of the whole menstrual cycle was used.

| | Phase of the cycle: | | |
|---|---|---|---|
| | Follicular | Periovulatory | Luteal |
| SERUM: | | | |
| LH | 0.06 | 0.13 | 0.29 |
| FSH | 0.71 | 0.47 | 0.16 |
| LH/FSH ratio | **0.02** | 0.05 | 0.09 |
| Prolactin | 0.07 | 0.36 | 0.40 |
| 17ß–Oestradiol | 0.26 | 0.73 | 0.25 |
| Progesterone | 0.37 | 0.47 | 0.59 |
| 17–OHP | 0.23 | 0.07 | 0.15 |
| A–dione | 0.16 | 0.60 | 0.70 |
| Testosterone | 0.05 | 0.09 | 0.21 |
| DHEA | 0.79 | 0.85 | 0.31 |
| DHEAS | 0.94 | 0.79 | 0.63 |
| Cortisol | 0.67 | 0.37 | 0.51 |
| SHBG | 0.46 | | |
| FAI | 0.88 | | |
| SALIVA: | | | |
| Oestradiol | 0.85 | 0.47 | 0.18 |
| Progesterone | 0.95 | 0.21 | 0.83 |
| 17–OHP | 0.40 | 0.80 | **0.02** |

| | Menstrual Cycle |
|---|---|
| SALIVA: | |
| A–dion | 0.08 |
| Testosterone | **0.003** |
| DHEA | **0.04** |
| DHEAS | 0.12 |
| Cortisol | 0.19 |

## 3.3 DISCUSSION

In the present investigation hormonal data were collected throughout one menstrual cycle of 15 infertile women with irregular cycles and 20 normally menstruating women.

Before the investigation all women of the study group had irregular cycles, either of too short (n=2) or of too long a duration (n=13). During the study the cycle lengths were within the normal range in seven women and were more than 32 days in eight women. This difference in cycle lengths before and during the investigation illustrates the irregular nature of the menstrual cycles of this group. In other investigations only a small variation within subjects was demonstrated, however, these referred to normally menstruating women.[2,3]

All study cycles were ovulatory as confirmed by an LH surge and postovulatory progesterone concentrations above 15 nmol/L.

The LH concentrations and LH/FSH ratios of the group with irregular cycles tended to be higher than in the control group, especially during the follicular and periovulatory phase of the cycle.

Furthermore the serum testosterone levels were in a higher range in the earlier phases of the cycle in the women with irregular cycles. The salivary androgens were also elevated in the group with irregular cycles in comparison to the normally menstruating women. It is reported that plasma testosterone levels are directly correlated with a significant prolongation of the follicular phase and that elevated androgen levels can indeed result in ovarian dysfunction.[4]

Some women in the control group also showed slightly elevated serum testosterone levels. These small elevations of testosterone were associated with slightly elevated serum DHEA and/or DHEAS concentrations and normal androstenedione concentrations. This indicates the source of androgen production to be the adrenals. In contrast, the elevated serum testosterone levels in the women with irregular cycles were mostly combined with elevations of androstenedione without an increase in serum DHEA and DHEAS concentrations. This finding suggests that the source of most androgen secretion in women with irregular cycles is the ovary.

Elevated LH and low or normal FSH values and excess androgen production are characteristic features of polycystic ovarian disease (PCOD).[5-7] In response to the elevated LH levels, theca and stroma cells secrete increasing amounts of androstenedione and testosterone. The aromatising capacity of C-19 steroids of the granulosa cell layer in polycystic ovaries is reduced due to insufficient FSH stimulation.[8] FSH deficiency and increased intraovarian androgen concentrations inhibit normal follicular maturation.[9]

However, the 17ß-oestradiol concentrations of the study group of women with irregular cycles were not different from those of the regularly menstruating females. Serum 17ß-oestradiol levels represent the sum of 17ß-oestradiol production derived from secretion by the ovary and from peripheral conversion of androgens to oestrogens. In the women of this study an increase in extraglandular oestradiol production may be compensated by a decrease of granulosa cell aromatising capacity.

In this study elevated LH levels and excess of androgen production have been demonstrated in women with irregular cycles. However, this hormonal status did not result in anovulation. It is possible that the women in this group suffer from a mild, subclinical or early form of PCOD characterised by infertility and endocrinological features of PCOD, but without anovulation. Also the classical manifestation of obesity was absent in the women of this study, since the Quetelet index was in the normal range and did not differ from those of the control group. The etiology of PCOD is still a matter of debate. It has been proposed by Schwartz et al[10] to consider PCOD as a gradually developing pathophysiological process. In this view the women in this study can be considered as having early forms of PCOD.

The biological activity of androgens is dependent on the amount of unbound, freely circulating hormone.[11,12] The level of SHBG is viewed as a major determinant in the control of the physiologically effective plasma levels of androgens and oestrogens. Testosterone is bound to SHBG and albumin for 99% and only 1% circulates freely in blood. Androgens suppress the production of SHBG, thus potentiating the biological activity of androgenic steroids.[13] The production of SHBG is, on the contrary, increased by oestrogens.

It has been reported that the free androgen index, i.e. the ratio of serum testosterone to SHBG, is a more sensitive parameter of hyperandrogenism.[14] However, the women with irregular cycles showed a wide range of SHBG concentrations and FAI's. In the women with irregular cycles as well as in the control group the FAI seemed to correlate with the saliva testosterone concentrations, although this relation was not significant. It is assumed that the concentration of a steroid in saliva represents the unbound, biologically active part of that hormone.[15,16] In this investigation no direct correlation was found between hormone determinations in serum and saliva, neither in the women with normal cycles, nor in the women with irregular cycles. The variation in the ratio serum to saliva concentrations of a steroid was rather large. Although not always at the same moment, all serum and saliva samples were obtained between 8:00 and 12:00 hour. Thus, the variation in saliva samples cannot be explained by a circadian rythm. The possibility should not be excluded that in pathological circumstances a discrepancy exists between the free fraction of a steroid, as represented by the steroid concentration in saliva, and the total amount of a steroid (serum concentration). This means that the proportion of unbound steroid in pathological circumstances can be different from that in normal conditions.

The cortisol levels of the women with irregular cycles were similar to those of the control women, which indicated the absence of hypercortisolism.

The plasma levels of progesterone and 17α-OH-progesterone, a derivate of progesterone, were stable during the follicular phase within the range of 2.5 to 3.0 nmol/L. A significant rise of both progestins was seen prior to the LH peak in serum and saliva. This is in agreement with other investigators who reported that an increasing secretion of progesterone and 17α-OH-progesterone just prior to the LH peak may indicate one of the earliest changes associated with luteinisation of the follicle and the approaching ovulation.[17-19] This parallel rise in progesterone and 17α-OH-progesterone appeared in

normally menstruating women and in the women with irregular cycles. The rise of progesterone persisted and reached maximal levels on day +6/+7 in the control group and on day +8/+9 in the group with irregular cycles. However, 17α-OH-progesterone concentrations showed a peak coincident with the LH peak with a small decline thercafter. Following this event a second peak was found during the midluteal phase. Periovulatory 17α-OH-progesterone concentrations tended to be higherin the women with cycle irregularities. The periovulatory 17α-OH-progesterone peak was also demonstrated in saliva. This small periovulatory 17α-OH-progesterone peak confirms other investigations.[20,21] However, these reports made no comment on the decline of 17α-OH-progesterone levels after the LH surge. Probably the LH peak itself or the event of the ovulation is the cause of the decline in 17α-OH-progesterone on day +1. It might be the expression of the termination of the $\Delta^5$ pathway of oestrogen synthesis and the initiation of the $\Delta^4$ pathway.

In both groups the progesterone levels tended to increase until later in the luteal phase and showed an abrupt decline there after, whereas 17α-OH-progesterone concentrations reached the maximum level earlier.

When comparing the prolactin levels of the group with irregular cycles with those of the control group, no statistical differences were found in any of the three periods of the cycle. A tendency existed towards higher prolactin concentrations in the follicular phases of the women with irregular cycles. In the women with irregular menstrual cycles a large variation was seen in prolactin levels both within one subject as well as between the subjects. This wide range of prolactin concentrations was most obvious during the luteal phase. It has been demonstrated that prolactin levels tend to be higher during the luteal phase than during the early follicular phase.[22-25] These differences in prolactin secretion probably reflect an altered hypothalamic neurotransmission during the luteal phase.[26]

Four women with irregular cycles can be assigned as hyperprolactinaemic, although not in all samples of their study cycles the prolactin concentration exceeded the upper limit of normal of 800 mIU/L. The pregnancies that were established in three of these four women very soon after initiation of bromocriptine treatment are not an absolute proof but very suggestive of an infertility associated with transient or intermittent elevated prolactin levels. Bromocriptine normalises elevated prolactin levels with concommittant restoration of ovarian function.[27]

More than 50% of the women with irregular cycles and 40% of the control group showed an elevated prolactin concentration at least once during the investigated period. On the other hand, the four women assigned as hyperprolactinaemic also showed occasionally prolactin concentrations within the normal range. This proves the need for at least two or three prolactin determinations in an infertility investigation program, as prolactin elevation can be intermittent. A single elevated level does not confirm pathology and a single normal value does not exclude it.

Excessive prolactin secretion is frequently associated with anovulation and amenorrhoea. Despite the increased prolactin levels found in both groups, the investigated cycles were all ovulatory. Even the four women assigned as hyperprolactinaemic had

ovulatory cycles during the study, and in one of them the cycle was of normal duration (31 days); the three other hyperprolactinaemic women were oligomenorrhoeic. None of the hyperprolactinaemic women showed any sign of a defective luteal phase as their luteal phases ranged from 14 to 17 days with progesterone levels within the considered normal luteal phase range.

The mechanisms by which prolactin exerts its effect on the fertility of these women is not fully clear. Secondary causes, such as hypothyroidism, medication, and renal or hepatic failure, have been excluded.

Hyperprolactinaemia can disturb the hypothalamic-pituitary-ovarian axis at several levels. Prolactin can act at the hypothalamic level by inhibiting the GnRH secretion and hence by disturbing the gonadotrophin release. Pituitary responsiveness to GnRH is normal in such women and a normal pattern of gonadotrophin pulsatile secretion is restored with small, repetitive doses of GnRH.[28] The baseline LH levels can be in the normal range in hyperprolactinaemic women, but without or with an altered pulsatility.[29] This can also result in anovulation and/or amenorrhoea. Leyendecker et al[30] demonstrated that ovulation can be induced in women with hyperprolactinaemic amenorrhoea when pulsatile LH secretion is re-established by the administration of intermittent exogenous LHRH, despite persistent elevation of prolactin concentrations.

At the pituitary level hyperprolactinaemia decreases the positive feedback of oestrogen on gonadotrophin release.31 However, ovarian response to exogenous gonadotrophin stimulation is maintained in hyperprolactinaemic amenorrhoea. Caro and Woolf[32] were able to show that the oestradiol elevation following the gonadotrophin surge induced by exogenous LHRH was not different in normal and hyperprolactinaemic women.) Hyperprolactinaemia can cause inhibition of ovarian activity by a direct inhibitory action of prolactin on the ovary. Prolactin can impair human luteal function. Some studies reported a shortening of the hyperthermic phase and a reduction in progesterone synthesis by the corpus luteum.[33-35] Other investigators, however, could not demonstrate this negative effect of prolactin on the luteal phase.[36]
Hyperprolactinaemic patients may represent heterogenous groups with different degrees of hypothalamic disorder. Probably the transient hyperprolactinaemia and the relatively mild elevation of prolactin in the women in this study was not sufficient to interfere with follicular maturation, ovulation, or corpus luteum function, but it may impair fertilisation and/or implantation. This confirms another report[37] which suggested that transient hyperprolactinaemia apparently does not disturb ovulation or luteal function but acts on targets other than the hypothalamic-pituitary-ovarian axis, such as ovum fertilisation or embryo implantation.

No direct relation could be demonstrated between the prolactin levels and the androgen levels. For every woman, including the four women with frequently elevated prolactin levels, the Kendall-coefficient of correlation was determined between prolactin and DHEAS on one hand and prolactin and testosterone on the other hand. No statistical correlation was found either between prolactin and DHEAS nor between prolactin and testosterone. Several studies have suggested an association between hyperprolactinaemia and high levels of DHEAS.[38-40] However, other investigators have

been unable to confirm this.[41,42] Even inverse relationships between prolactin levels and both DHEAS and testosterone levels have been reported.[43]

In summary, the observations presented provide a detailed picture of the hormonal events in serum and saliva in women with normal and in women with irregular menstrual cycles. Menstrual irregularity cannot be regarded as a pathophysiological entity and ovulatory dysfunction may be caused by different etiologic factors. The fifteen women who formed the study group showed relatively small differences in endocrinological parameters to the control group. However, two major cycle disturbances could be recognised: early or mild forms of PCOD and transient hyperprolactinaemia. I tcan therefore be concluded that the evaluation of irregular menstrual cycles must be directed to the diagnosis or exclusion of these disturbances. For the detection of hyperandrogenism salivary testosterone is a usefull tool. Since transient hyperprolactinaemia may be difficult to diagnose, repeated sampling should be performed during the luteal phase before a conclusion can be drawn whether this is present or not.

This study also shows the existence of a gradual transition from normal menstrual cycles through mild PCOD or transient hyperprolactinaemia to obvious cycle disturbances such as amenorrhoea caused by frank PCOD or hyperprolactinaemia.

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# CHAPTER 4

# ENDOCRINOLOGICAL PARAMETERS OF THE GROUP WITH UNEXPLAINED INFERTILITY COMPARED TO THE CONTROL GROUP

## 4.1 INTRODUCTION

The diagnosis unexplained infertility is established in couples in whom complete and intensive investigation and up to date medical examination fail to reveal any cause for their childlessness. The better the understanding of the physiology of reproduction and the more advanced the diagnostic approach, the less frequently this unsatisfactory diagnosis has to be used. (see for review 1-4) The incidence of unexplained infertility depends on the limits of evaluation and medical knowledge. During the last 20 years considerable progress has been made in the diagnosis of causes of infertility. Therefore the incidence of unexplained infertility has declined from approximately 80% of infertile couples to 5% to 10%.

The incidence also depends on the criteria used to define normality and abnormality. Sometimes infertility despite correction of the factor(s) identified as responsible for infertility, (e.g. normalisation of the menstrual cycle by ovulation inducing drugs or treatment of endometriosis) is considered as unexplained infertility.[2]

The results of various, often empirical, therapies (e.g. clomiphene citrate[5] and bromocriptine[6]) used for these couples suggest abnormalities in the endocrine patterns of the cycle. However, the results of such studies are of little value if not compared with the pregnancy rates in series of untreated couples in a prospective manner, since the spontaneous pregnancy rate for couples with unexplained infertility without any treatment is reported to be 36% to 87%.[7-9]

Subtle endocrinological disorders have been detected in women in whom no cause for infertility could be found.[10-15] Such disorders, if present, may only be detected by detailed endocrine profiles. In this study an extensive endocrinological assessment was performed in women from couples with unexplained infertility after the routine screening procedure. Because of the uncertainty about the limits of normal variation of the different hormones and the ranges which can be considered with confidence to indicate a normal ovulatory cycle, the results were compared with the hormonal data obtained in women with presumed normal fertility.

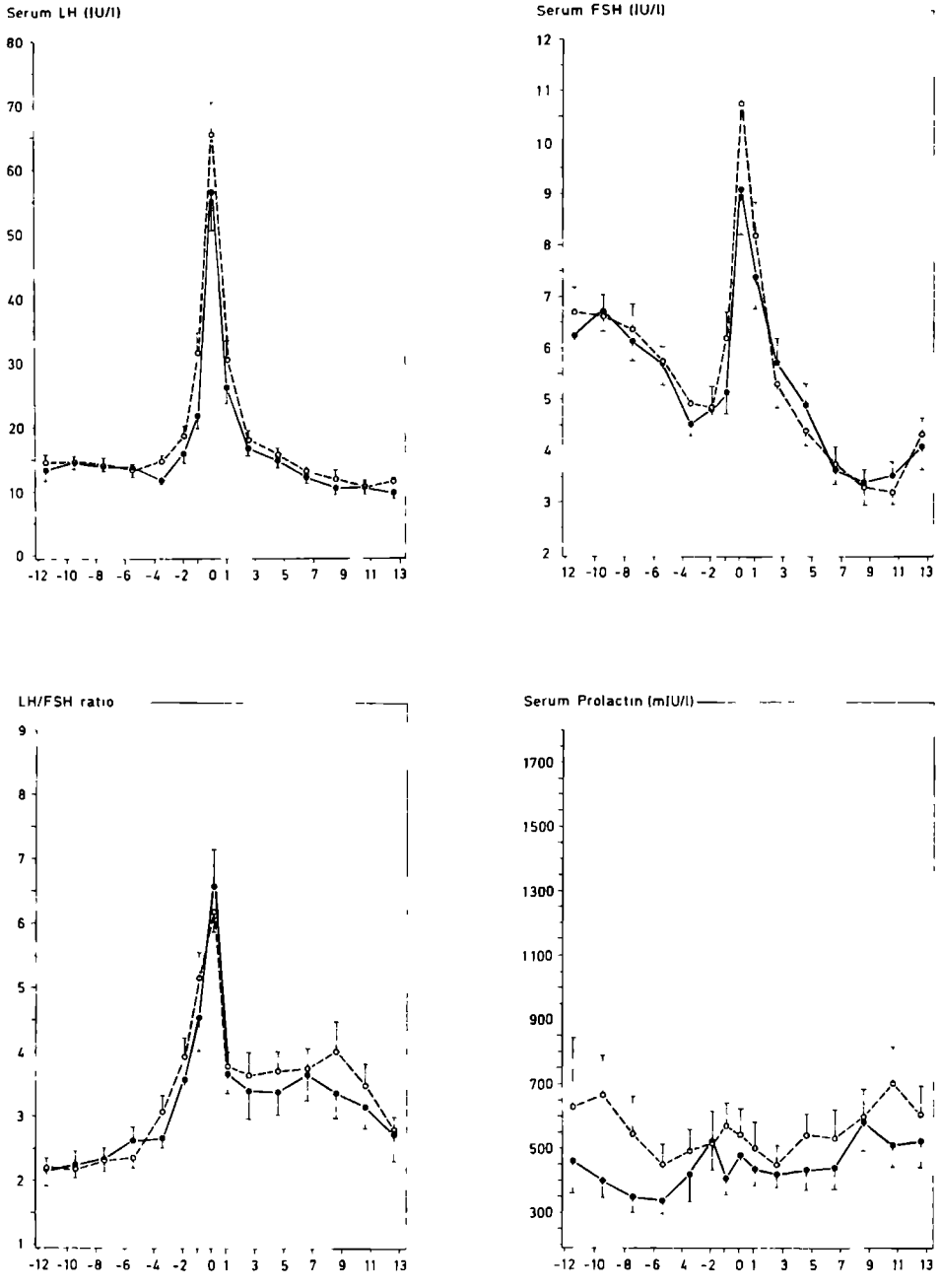


**Figure 4.1** Serum levels of LH, FSH, LH/FSH ratio and prolactin during one menstrual cycle of 20 women with unexplained infertility (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

## 4.2 RESULTS

### 4.2.1 Endocrinological parameters in serum

*4.2.1.1 LH, FSH and the LH/FSH ratio (Fig 4.1)*

Figure 4.1 shows the patterns of LH, FSH and the LH/FSH ratio of the group with unexplained infertility (dotted line) and the control group (drawn line). The LH concentrations were significantly higher during the follicular phase in the women with unexplained infertility in comparison to the control group ($p=0.02$). During the periovulatory phase also a tendency existed to higher LH concentrations in the group with unexplained infertility ($p=0.09$). An earlier onset of the LH surge in the women with unexplained infertility is seen in figure 4.1 in comparison to the control group. No statistically significant difference in LH levels between the two groups was found in the luteal phase of the cycle.

The FSH levels and the LH/FSH ratios of the group with unexplained infertility were not statistically different from those of the control group.

In both groups the LH levels during the luteal phase were higher than during the follicular phase, whereas the FSH levels were lower during the luteal phase.

*4.2.1.2 Prolactin (Fig 4.1)*

No statistical difference was found in prolactin levels between the group with unexplained infertility and the control group.

**Individual observations**

Eleven women with unexplained infertility and also eleven fertile women showed incidentally an increased prolactin level (>800 mIU/L). However, no women of the fertile group showed prolactin levels above 1500 mIU/L, whereas in five women with unexplained infertility prolactin levels exceeded 1500 mIU/L at least once. Six women with unexplained infertility showed elevated prolactin concentrations on four or more occasions during the investigation. All women with transient elevated prolactin levels had a normal cycle length during the investigation. The mean prolactin concentration of the investigated cycle of three women with unexplained infertility exceeded the upper limit of normal in our laboratory of 800 mIU/L. Three women with transient hyperprolactinaemia became pregnant, one of whom after bromocriptine treatment.

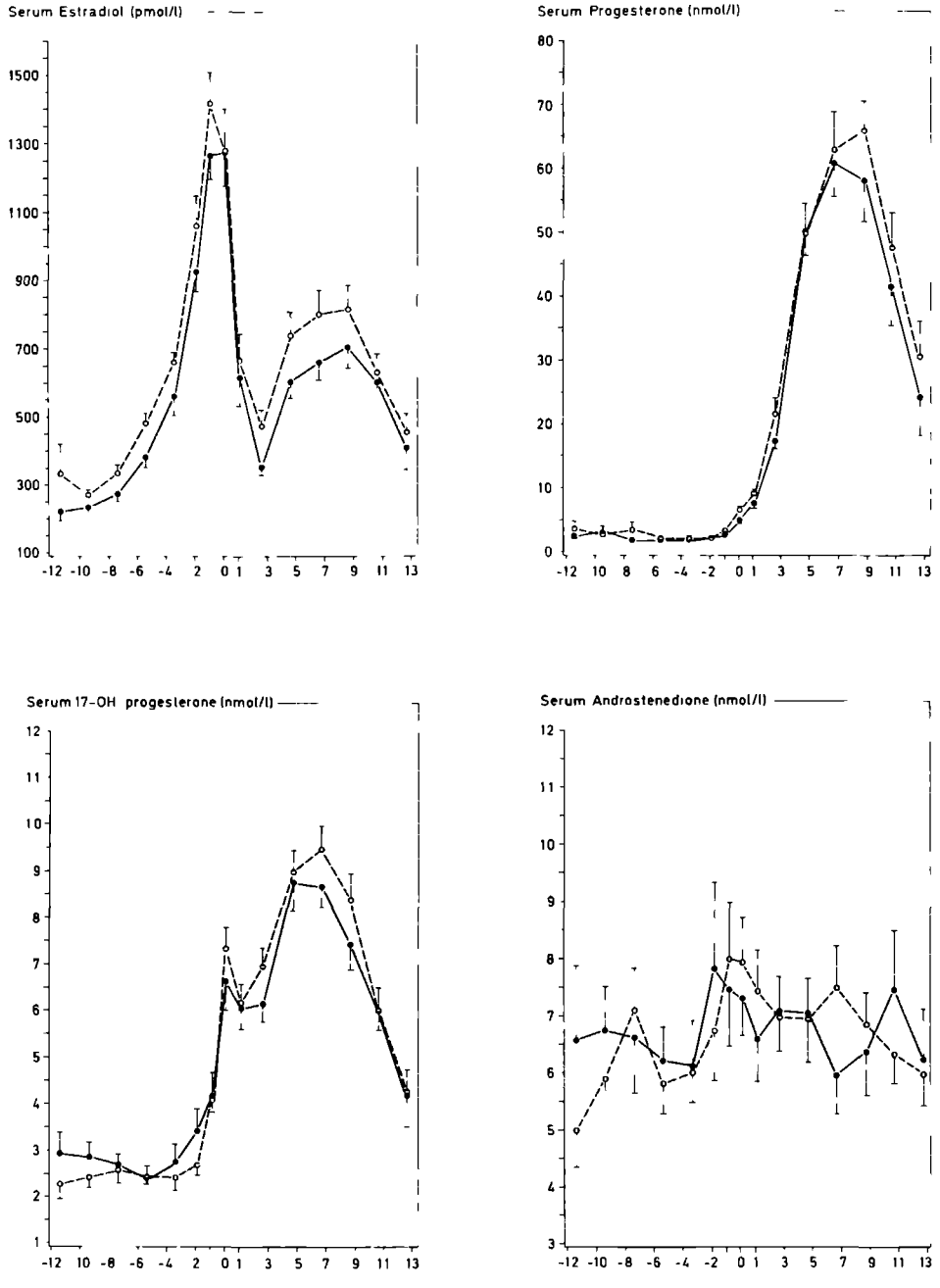


**Figure 4.2** Serum levels of 17ß-oestradiol, progesterone, 17α-OH-progesterone and androstenedione during one menstrual cycle of 20 women with unexplained infertility (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

*4.2.1.3 17β-Oestradiol (Fig 4.2)*

The 17ß-oestradiol levels were higher during the follicular phase in the women with unexplained infertility (p=0.04).

The cyclical pattern of 17ß-oestradiol in the group with unexplained infertility was similar to that of the control group with a maximum concentration on cycle day -1 or 0 and a luteal peak on cycle day +8/+9.

*4.2.1.4 Progesterone (Fig 4.2)*

The progesterone levels were not statistically different in either of the three phases of the cycle. The first significant rise of progesterone was on the day previous to the LH peak (cycle day -1) in both groups. The progesterone peak was on day +8/+9 in the women with unexplained infertility and on day +6/+7 in the control group.

*4.2.1.5 17α-OH-Progesterone (Fig 4.2)*

The 17α-OH-progesterone levels of the two groups were in the same range and were not statistically different. The first significant increase in 17α-OH-progesterone concentrations advanced the LH surge by one day in both groups. Also a small 17α-OH-progesterone peak was seen on day 0 in both groups, and a second peak in the luteal phase preceding the progesterone peak.

*4.2.1.6 Androstenedione (Fig 4.2)*

The androstenedione levels of the group with unexplained infertility were significantly higher only during the luteal phase (p=0.05). No clear cyclical pattern for androstenedione was seen in either of the two groups. When the women were considered individually, the highest concentration of androstenedione was usually found during the periovulatory period.

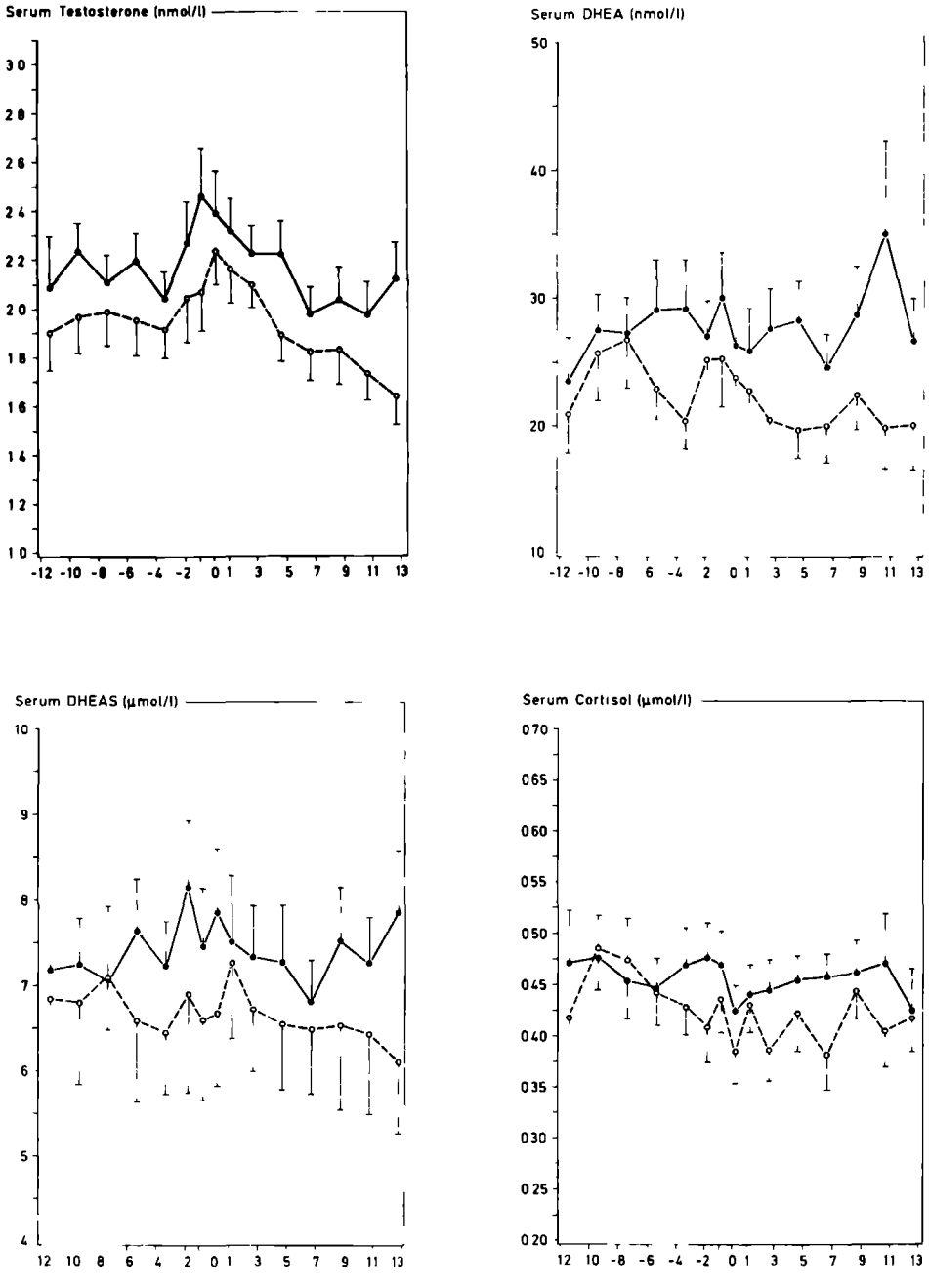


**Figure 4.3** Serum levels of testosterone, DHEA, DHEAS and cortisol during one menstrual cycle of 20 women with unexplained infertility (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

*4.2.1.7 Testosterone (Fig 4.3)*

Figure 4.3 suggests lower testosterone levels in the women with unexplained infertility compared to the control group, but this difference was not statistically significant.

In the individual woman of both groups the highest testosterone concentration was seen usually during the periovulatory phase and the lowest concentration during the luteal phase.

*4.2.1.8 DHEA and DHEAS (Fig 4.3)*

During the luteal phase DHEA levels tended to be lower in the women with unexplained infertility in comparison to the control group (p=0.09). No cyclical pattern in DHEA and DHEAS concentrations was seen.

*4.2.1.9 Cortisol (Fig 4.3)*

No differences between the two groups were found in serum cortisol levels. The cortisol concentrations did not differ throughout the menstrual cycle.

*4.2.1.10 SHBG and the FAI*

In one woman with unexplained infertility and four fertile women SHBG was determined throughout the whole cycle: there was no obvious cyclical pattern. In the other patients SHBG was determined in the very early follicular phase. SHBG concentrations in the group with unexplained infertility ranged from 30 to 130 nmol/L and from 34 to 93 nmol/L in the control group. The median concentrations were 69 and 50 nmol/L respectively.

The FAI, i.e. the ratio of total serum testosterone to SHBG x 100, ranged from 1.28 to 9.12 in the group with unexplained infertility and from 1.51 to 10.3 in the control group. The median FAIs were 2.76 and 3.90 respectively.

**Individual observations**

Two women with unexplained infertility with mean serum testosterone concentrations of 2.49 and 2.87 nmol/L (high-normal) respectively, and SHBG levels of 30 and 34 nmol/L (low-normal) and high FAIs (8.0 and 9.12 respectively), had high serum DHEA and DHEAS concentrations throughout the whole cycle.

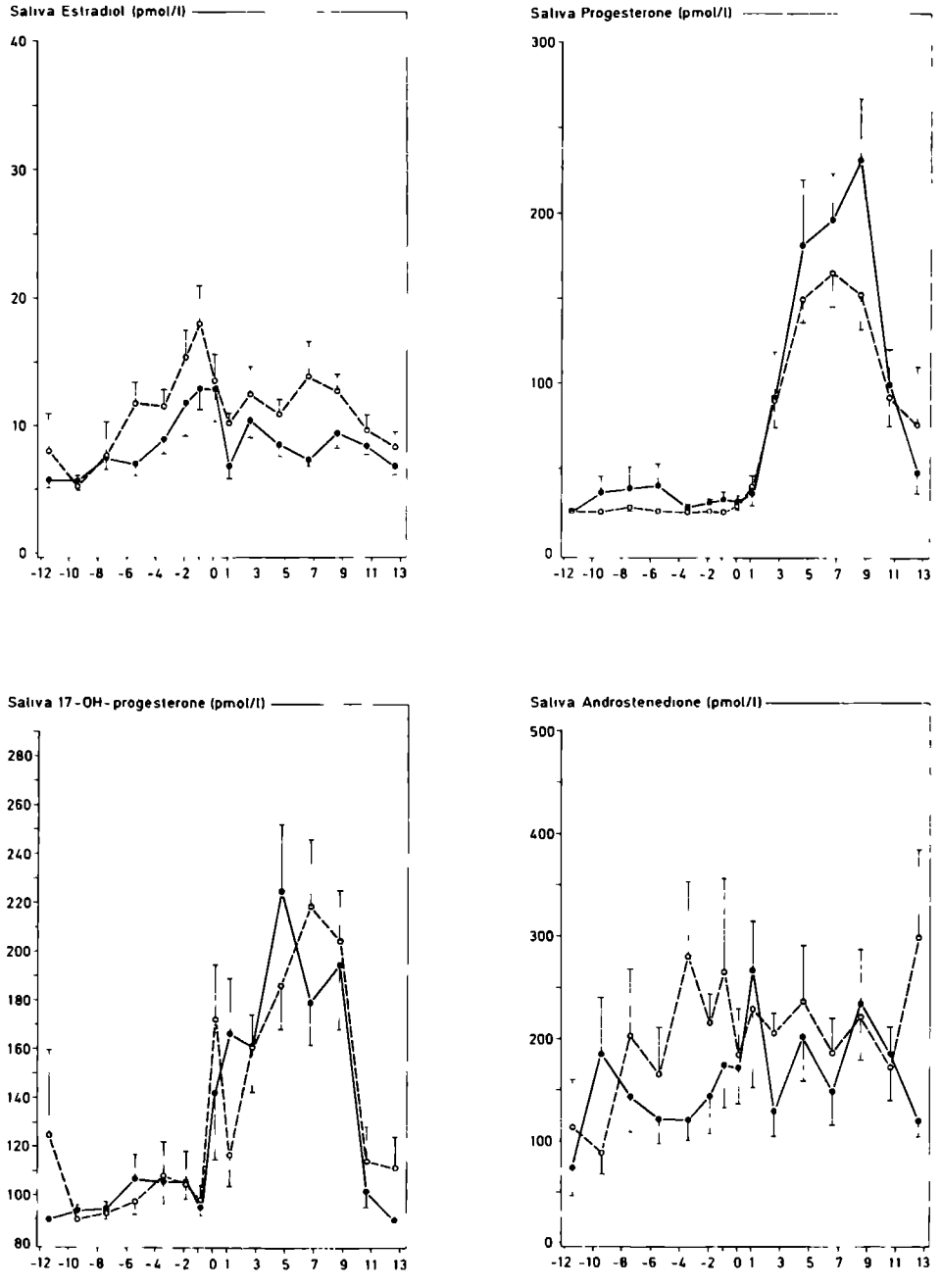


**Figure 4.4** Saliva levels of 17ß-oestradiol, progesterone, 17α-OH-progesterone and androstenedione during one menstrual cycle of 20 women with unexplained infertility (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

### 4.2.2 ENDOCRINOLOGICAL PARAMETERS IN SALIVA

*4.2.2.1 17ß-Oestradiol (Fig 4.4)*

The concentrations of 17ß-oestradiol in saliva were raised in the women with unexplained infertility compared to the fertile women (follicular phase: p=0.03, periovulatory phase: p=0.15 and luteal phase: p=0.004).

The maximum concentration of saliva 17ß-oestradiol was found on cycle day -1 in both groups. Only in the group with unexplained infertility a second peak appeared on day +6/+7 of the luteal phase.

*4.2.2.2 Progesterone (Fig 4.4)*

There was insufficient evidence for a significant difference in saliva progesterone levels between the two groups. The progesterone levels in saliva in the women with unexplained infertility tended to be lower during the periovulatory period (p=0.07). In both groups a cyclical pattern was seen similar to that of progesterone in serum. However, no significant increase of progesterone previous to the LH peak was recognised in saliva.

*4.2.2.3 17α-OH-Progesterone (Fig 4.4)*

No statistical difference was found in 17α-OH-progesterone levels between the group with unexplained infertility and the control group. The cyclical pattern in saliva was similar to that in serum with a clear midluteal peak concentration. In the women with unexplained infertility also a peak was found on day 0, like in serum. In the control group a periovulatory peak was also detected, but the maximum concentration was detected on the day after the LH peak, day +1.

*4.2.2.4 Androstenedione (Fig 4.4)*

Neither a difference between the two groups, nor a cyclical pattern was found for the saliva androstenedione concentrations.

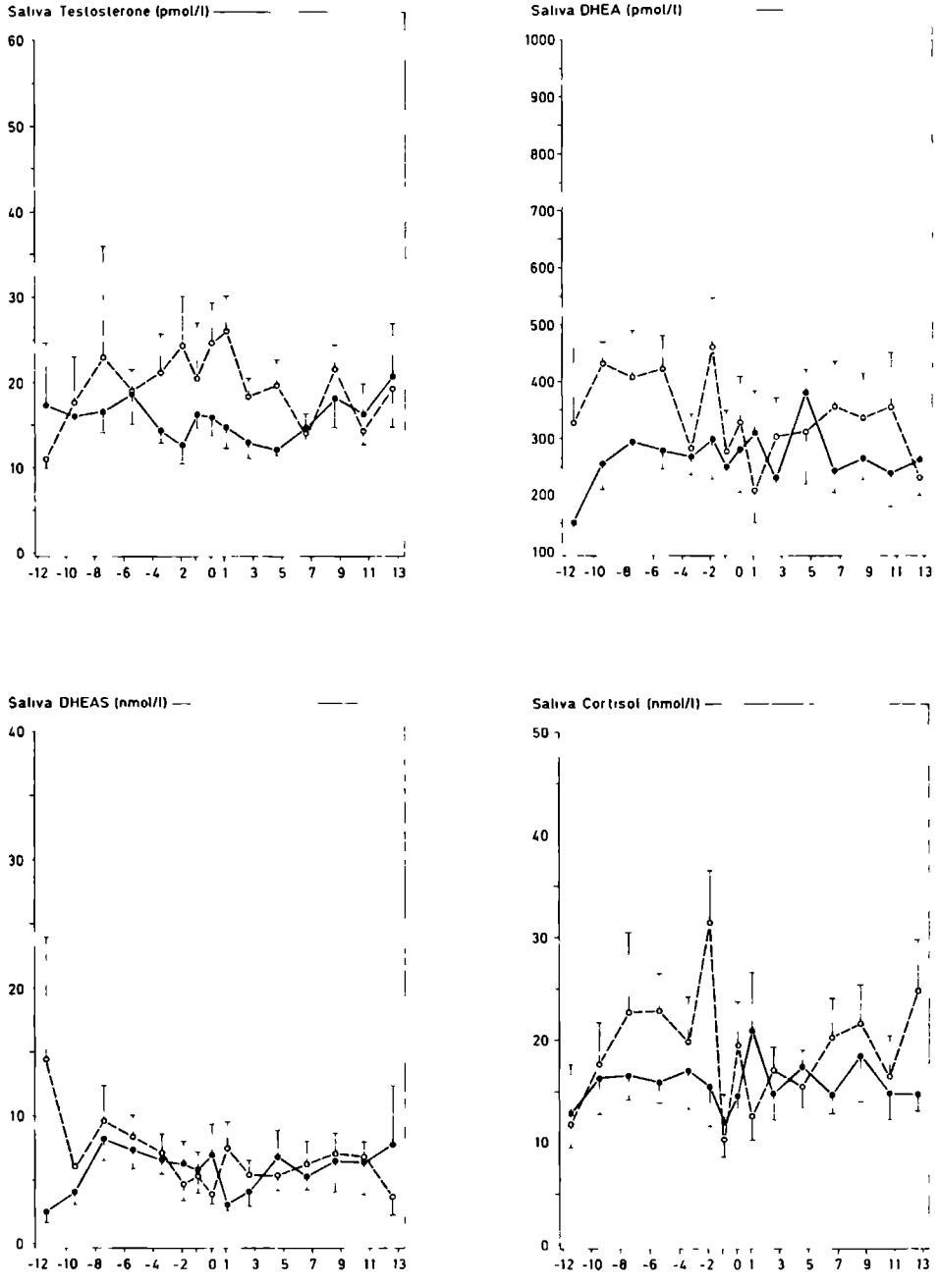


**Figure 4.5** Saliva levels of testosterone, DHEA, DHEAS and cortisol during one menstrual cycle of 20 women with unexplained infertility (dotted line) and 20 fertile women (drawn line). (Mean ± SEM)

*4.2.2.5 Testosterone (Fig 4.5)*

The saliva concentrations of testosterone of the women with unexplained infertility tended to be higher in comparison to the fertile women (p=0.06). Neither in the control group nor in the group with unexplained infertility a cyclical pattern could be recognised.

*4.2.2.6 DHEA and DHEAS (Fig 4.5)*

The levels of DHEA and DHEAS in saliva were not different for the two groups.

*4.2.2.7 Cortisol (Fig 4.5)*

Cortisol concentrations in saliva were in the same range in women with unexplained infertility and fertile women.

**Table IV.1** P-values of the comparison of the collected hormonal data of the group with unexplained infertility versus the control group. (statistical test described by Koziol).
For comparison of 17-OHP, A-dione, testosterone, DHEA, DHEAS and cortisol in saliva the median value of the whole menstrual cycle was used.

| | Phase of the cycle: | | |
|---|---|---|---|
| | Follicular | Periovulatory | Luteal |
| SERUM: | | | |
| LH | **0.02** | 0.13 | 0.57 |
| FSH | 0.38 | 0.42 | 0.72 |
| LH/FSH ratio | 0.43 | 0.77 | 0.53 |
| Prolactin | 0.18 | 0.87 | 0.68 |
| Oestradiol | **0.04** | 0.69 | 0.16 |
| Progesterone | 0.31 | 0.30 | 0.61 |
| 17-OHP | 0.48 | 0.82 | 0.25 |
| A-dione | 0.95 | 0.73 | 0.05 |
| Testosterone | 0.51 | 0.65 | 0.09 |
| DHEA | 0.21 | 0.77 | 0.09 |
| DHEAS | 0.65 | 0.11 | 0.24 |
| Cortisol | 0.67 | 0.42 | 0.35 |
| SHBG | 0.27 | | |
| FAI | 0.32 | | |
| SALIVA: | | | |
| Oestradiol | **0.03** | 0.15 | **0.004** |
| Progesterone | 0.18 | 0.07 | 0.27 |
| 17-OHP | 0.61 | 0.82 | 0.56 |
| | Menstrual Cycle | | |
| SALIVA: | | | |
| A-dione | 0.27 | | |
| Testosterone | 0.06 | | |
| DHEA | 0.67 | | |
| DHEAS | 0.56 | | |
| Cortisol | 0.34 | | |

## 4.3 DISCUSSION

Serum and saliva hormonal parameters of 20 women with unexplained infertility were collected during one menstrual cycle. The data were compared with a control group. Normal main parameters in a fertility workup program were used to select the group with unexplained infertility. It can be expected that this group is not homogeneous, since many infertility problems, both of male and female, are not fully elucidated. In the case of unexplained infertility minor abnormalities in either male or female reproductive function can very well be present.

In spite of the selection criterion of regular menstrual cycles of normal duration between 26 and 34 days, three women had a cycle length of 24 days and two women had an oligomenorrhoea with an interval of 39 and 63 days, respectively, during the investigated period. Fifteen women with unexplained infertility had a normal cycle length during the same period.

All women suffering from unexplained infertility had an ovulatory cycle, as could be confirmed both by sufficiently elevated progesterone levels during the luteal phase and by ultrasound monitoring of the cycle. In all females follicle growth and the disappearance of the follicle was seen ultrasonographically. This finding makes a luteinised unruptured follicle (LUF) syndrome very unlikely. Luteinisation of an unruptured follicle is characterised by normal endocrinologic patterns and a biphasic BBT. Failure of follicle rupture has been demonstrated to occur more frequently in unexplained infertility.[16]

In the extensive investigation of the hormonal characteristics of the females of couples with unexplained infertility a wide range of individual prolactin concentrations was found, which was not clearly related to the stage of the cycle or to any other of the reproductive hormones. Six women demonstrated elevated prolactin levels on four or more occasions. These females can be considered as having intermittent or transient hyperprolactinaemia. The pregnancies that resulted after treatment of transient hyperprolactinaemia with bromocriptine, as described in chapter 3 and also in one woman with unexplained infertility treated with bromocriptine, indicate the possibility of transient hyperprolactinaemia as a cause of infertility.

Hyperprolactinaemia as a cause of, or in association with, amenorrhoea and anovulation has been well documented.[17] In contrast, consistently elevated prolactin levels or transient hyperprolactinaemia in combination with regular, ovulatory menstrual cycles have also been reported.[18-22] However, prolactin concentrations have been shown to be significantly lower in conception than in non-conception cycles.[23] The effect of transient hyperprolactinaemia on menstrual cycle and on fertility and the importance of this phenomenon are not exactly known.

Infertility in normally menstruating women associated with hyperprolactinaemia has been attributed to luteal phase deficiency, which is a reflection of a follicular disorder.[24] According to Nakano[18] hyperprolactinaemia in infertile women with regular menstrual cycles was associated with defects in follicle development, as measured by oestradiol production during the mid-follicular phase, but not with reduced corpus luteum

function. Glazener et al.[21] also found no evidence of luteal phase insufficiency in infertile women with normal menstrual cycles and hyperprolactinaemia. Furthermore, the chance of conception was not related to the prolactin levels. The women with transient elevated prolactin levels in this investigation also showed no evidence of luteal phase insufficiency as assessed by luteal progesterone concentrations and the duration of the luteal phase.

High prolactin levels may also be due to the presence of immunoreactive molecular forms that are biologically inactive *in vivo*, such as 'big,big' prolactin.[25] The molecular heterogeneity may be the explanation for the absence of pathological effects in some patients with apparent elevated levels of serum prolactin.

Bromocriptine has been used to treat women with unexplained infertility,[6] but one controlled study has denied any affect.[26] Ben-David and Schenker[27] found in 94% of women with regular menses and longstanding idiopathic infertility a transitory elevation of their serum prolactin levels. Treatment with bromocriptine resulted in a pregancy rate of 40%. They concluded that this transitory, relatively mild hyperprolactinaemia was not sufficient to interfere with follicular maturation, ovulation, or corpus luteum function, but it would impair fertilisation and/or implantation. Transient hyperprolactinaemia may be considered as the presentation of an early phase in the natural evolution of hyperprolactinaemic amenorrhoea.[20]

It also follows from these observations that in all infertile women, including those with unexplained infertility, prolactin concentration should be determined on at least two occasions.

The usefulness of treatment of this abnormality is not clear. Properly controlled prospective therapeutic trials with dopamine agonistic drugs like bromocriptine in normally menstruating women with transient hyperprolactinaemia are required.

The LH levels of the women with unexplained infertility were higher compared to the LH concentrations of the control group during the follicular phase. During the periovulatory phase a tendency existed towards higher LH levels in the women with unexplained infertility. The preovulatory rise of LH concentrations started earlier in the women with unexplained infertility in comparison to the control group. Serum FSH levels and the LH to FSH ratios did not differ between the two groups. The normal FSH levels in the women with unexplained infertility provide the development of follicular growth, but the accelerated onset of the LH surge may result in an early preovulatory luteinisation of the follicle. The early luteinisation of the follicle is not confirmed by serum progesterone levels, but periovulatory levels of saliva progesterone tended to be elevated in the women with unexplained infertility. A similar observation was reported from a study of PCOD patients treated with LHRH, who had elevated LH levels and normal FSH levels in the follicular phase and both normal LH and FSH concentrations during the luteal phase.[28]

Another explanation for the contribution of high follicular phase LH concentrations to infertility may be a direct effect on maturation of the oocyte itself. A significant reduction in the fertilisation rate and failure of implantation of embryos in an *in vitro*

fertilisation and embryo transfer program was observed in women who demonstrated elevated LH levels in their follicular phase.[29]

The 17ß-oestradiol concentrations of the group with unexplained infertility were also elevated in the follicular phase of the study cycle. Furthermore, saliva 17ß-oestradiol levels were statistically significantly elevated during the follicular and luteal phases of the investigated cycle. The elevated 17ß-oestradiol values can be interpreted as a result of the elevated LH concentrations. In response to the increased LH levels, the thecal cells are stimulated to produce androgens, which can then be converted to oestrogens in the granulosa cells, through FSH-induced aromatisation. The testosterone concentrations in saliva, which correlate well with the free fraction of testosterone,[30] tended to be higher in the women with unexplained infertility. The excessive free testosterone concentrations serves as substrate for peripheral conversion of testosterone to 17ß-oestradiol by extra-glandular tissue.

The high saliva 17ß-oestradiol concentrations, which are supposed to represent the free, biologically active part of 17ß-oestradiol,[31-33] detected in the women with unexplained infertility may inhibit ovum transport after fertilisation and thereby causing infertility. In the hamster it was observed that oviductal contractility is related to oestradiol and through that influences ovum transport to the uterus.[34] Contractions of the oviduct start as soon as the 17ß-oestradiol levels decline.

After IVF and ET procedures it was demonstrated that the progesterone to oestrogen ratio during the luteal phase was a better predictor of implantation than the absolute concentration of either hormone.[35-37] In mice implantation was inhibited by relatively high levels of 17ß-oestradiol.[36]

Serum concentrations of androstenedione, testosterone and DHEA tended to be lower during the luteal phase of the cycles of the women with unexplained infertility. Nevertheless, the testosterone concentration in saliva of the women with unexplained infertility tended to be higher throughout the investigated cycle. Measurement of salivary testosterone gives a useful indication of levels of biologically available androgen.[38] A highly significant correlation between the concentration of testosterone in saliva and the concentration of unbound testosterone in plasma has been demonstrated, both in normal and in hyperandrogenic patients.[39] However, Wang et al.[40] reported that the free testosterone index (= the free androgen index) was a better discriminator of hyperandrogenism.

The increased secretion of LH, the elevated testosterone concentrations in saliva and the subsequent increased levels of 17ß-oestradiol in women with otherwise unexplained infertility, may be associated with a suboptimal menstrual cycle that prevents the initiation of a pregnancy.

Despite two normal semen analyses before entrance to the investigation, six men of couples with unexplained infertility had a slightly abnormal SA and four men showed severe abnormalities in their SA. In the control group nine SAs were performed and four men had an abnormal spermiogram. No uniform agreement exists about the lower limits of a normal semen analysis. It is not certain when a semen sample, presumed to be normal, is also fertile. From several studies it is concluded that there is a significant

overlap between the sperm characteristics of men known to be fertile and those presumed to be infertile.[41-44] The combination of all variables of the standard SA has a better predictive value in the investigation of infertility than each of the separate characteristics.[43] Low values, however, provided stronger indication of poor fertility. Standard semen analysis does not necessarily describe function. At present there is no simple *in vitro* method by which the *in vivo* fertilising capacity of sperm can be reliably measured. The SPA in this study also provided no absolute criterium of fertility and infertility, since only four men in the fertile group (n=8) had a positive SPA. Fourteen males of couples with unexplained infertility had a positive SPA. Eight males of the group with unexplained infertility had a completely normal SA, a sufficient motility after preincubation and a positive SPA. The remaining twelve men had at least one abnormality in the investigations of the semen sample. No absolute conclusions about one's fertility potential can be drawn from the results of the conventional SA and the SPA, since both fertile men demonstrated abnormalities in SA and SPA and infertile men can have a normal SA and SPA.

The criteria for the diagnosis of unexplained infertility as used in our clinic closely resemble that of the definitions proposed by others. Despite this, 90% (18/20) of the couples classified as having unexplained infertility before entrance into the investigation, showed, after extensive investigation, subtle abnormalities in the female's hormonal secretory pattern and/or in the male's investigation of the ejaculate. It is, however, uncertain wether these abnormalities are responsable for their infertility.

This study shows once more how unsatisfactory it is to diagnose an infertility as unexplained. With intensive investigations of the hormonal parameters at the proper moments in the menstrual cycle and with repeated, detailed and well standardised analysis of the semen propably 90% of the so-called unexplained infertility can be explained. It is to be hoped and expected that the remaining 10% will disclose an explanation in the near future.

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# CHAPTER 5

# HUMAN CHORIONIC GONADOTROPHIN (hCG) MEASUREMENTS IN THE LUTEAL PHASE OF THE MENSTRUAL CYCLE OF INFERTILE AND FERTILE WOMEN

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## 5.1 INTRODUCTION

There is evidence that women trying to conceive have a high incidence of early conceptual loss in the luteal phase of the menstrual cycle. In 1952 Hertig et al.[1] examined 34 fertilised human ova recovered during the first 17 days after conception from uteri removed from fertile women. They found that 13 fertilised ova (38%) were morphologically abnormal and could not have resulted in a normal pregnancy. On the basis of a statistical model Roberts and Lowe[2] postulated that most of the human conceptions are lost and only 22% of the conceptions lead to the birth of a child. Biggers[3] estimated the probabilities of conception, recognisable pregnancy and live birth. He calculated a probability of fertilisation of an ovum of 0.84, and a probability of a recognisable pregnancy of 0.42. By the time pregnancy is recognised, half the embryos are lost. The probability of a live birth is 0.31.

Other studies[4-7] reported on the presence of human chorionic gonadotrophin (hCG) in the luteal phase of the menstrual cycle as the only evidence of pregnancy without a delay of the menstrual period. These studies all related to normally fertile women. It is not clear yet whether unrecognised pregnancy wastage occurs in women with unexplained infertility and it is unknown whether this phenomenon is of importance as a cause of unexplained infertility.

The purpose of the present study is to investigate the possible occurrence of early conceptual loss in women with unexplained infertility by measuring hCG levels in serum during the luteal phase. The results are compared with the levels of a control group of assumed fertile women.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Selection of females with unexplained infertility

Twenty women of couples suffering from unexplained infertility with a duration of 12 months or more formed the study group. The mean duration of their infertility at the time of the investigation cycle was 37.5 months (SD 19.4, range 12 - 72 months). They had regular menstrual cycles of 26 - 32 days with biphasic BBT charts and a luteal

phase of at least 12 days with sufficiently elevated progesterone levels. The husband's semen analysis was normal on at least two occasions (≥20 x $10^6$ sperm cells per mL, ≥50% with good forward progression and ≤40% abnormal forms). The postcoital test was positive and no abnormalities were demonstrated during hysterosalpingography and /or during laparoscopy. None of the patients received treatment during this study.

### 5.2.2 Selection of the control group

The control group consisted of 20 volunteer women who were either parous or who had no reason to doubt their fecundity and who were attempting to conceive. Conditions for admission in the control group were regular menstrual cycles of 26 to 32 days, no pregnancy during 3 months preceding the study cycle, trying to conceive during at most 6 months and no suspicion on pathology interfering with fertility. The groups were comparable regarding age.

### 5.2.3 Detection of ovulation

Follicular growth and ovulation was monitored by ultrasound every day from day 10 of the menstrual cycle onwards. The day a follicle of at least 18 mm had disappeared was supposed to be the day of ovulation. In the group with unexplained infertility daily serum LH measurements took place. The day after the maximal LH level (LH-peak) was supposed to be the day of ovulation. The day of ovulation was called day 0.

### 5.2.4 Schedule of blood sampling

Blood samples were collected by vena puncture and were taken every other day starting on the $4^{th}$ or $5^{th}$ day after ovulation. Sampling was continued until the next menstruation or until pregnancy was confirmed.

### 5.2.5 Determinations of hCG

Three different immunoassay procedures were used: a two-component Radio-ImmunoAssay (RIA) for hCG which detects both intact hCG and the free ß-subunit of hCG ('total hCG'), a free hCG-ß specific RIA ('free hCG ß-subunit') and an Immuno EnzyMetric Assay (IEMA) specific for the intact hCG molecule ('intact hCG'). A survey of the characteristics of the three assays is given in table V.1. The two RIAs have been developed in our laboratory and have been described earlier.[8] The IEMA is a commercially available assay system (Tandem-E hCG, Hybritech Europe S.A., Liege, Belgium) using monoclonal antibodies.

**Table V.1** Analytical characteristics of the hCG immunoassay systems

| Assay | Reference | Results | Analytical sensitivity | Clinical sensitivity | Intra-assay coefficient of variation | Inter-assay coefficient of variation |
|---|---|---|---|---|---|---|
| Total hCG | 1st IRP | ng/mL* | 0.7 | 2.0 | 7.3 | 12.0 |
| Free hCG ß-subunit | 1st IRP-ß | mIU/L** | 50 | 100 | 6.3 | 9.3 |
| Intact hCG | 2nd IS, 1st IRP | IU/L | 2.5 | 2.5 | 3.3 | 6.3 |

* 1 ng/mL = 15 IU/L (1st IRP-hCG) = 0.23 IU/L (1st IRP-hCG-ß)
2 ng/mL = 34 IU/L (1st IRP-hCG) = 0.41 IU/L (1st IRP-hCG-ß)

** 1st IRP-hCG-ß: 1ng = 1mIU, by definition

## 5.3 RESULTS

None of the women with unexplained infertility became pregnant during the investigation. The duration of their luteal phases (from 11 to 17 days) was in the normal range.

Five presumed fertile women became pregnant during the study cycle and delivered all from healthy children. The remaining 15 women of this group menstruated on the 12th to the 16th day after ovulation.

### 5.3.1 Total hCG

Three patients of the group of unexplained infertility demonstrated detectable serum total hCG during the luteal phase. The first woman showed this on the 16th day after ovulation, the day her menstrual period started; the second woman on days 6, 9 and 13 after ovulation and the third woman on the 8th and 10th day after ovulation. In this group no woman achieved a clinical pregnancy and no total hCG values were found above 1.9 ng/mL.

In 18 of the 20 fertile women total hCG was detected at least on one occasion in the luteal phase. In 13 of these 20 assumed fertile women elevated total hCG values were found in the luteal phase followed by a normal menstruation (table V.2). Five fertile women achieved a clinical, ongoing pregnancy (fig 5.1). These were all singleton pregnancies, confirmed by ultrasound. In two females no hCG levels were found above 1 ng/mL.

The elevated total hCG values showed no consistent pattern. In two women total hCG was already detected on the 4th day after ovulation. In one woman a total hCG level of 2.8 ng/mL was measured on the 15th day, the same day her menstruation began.

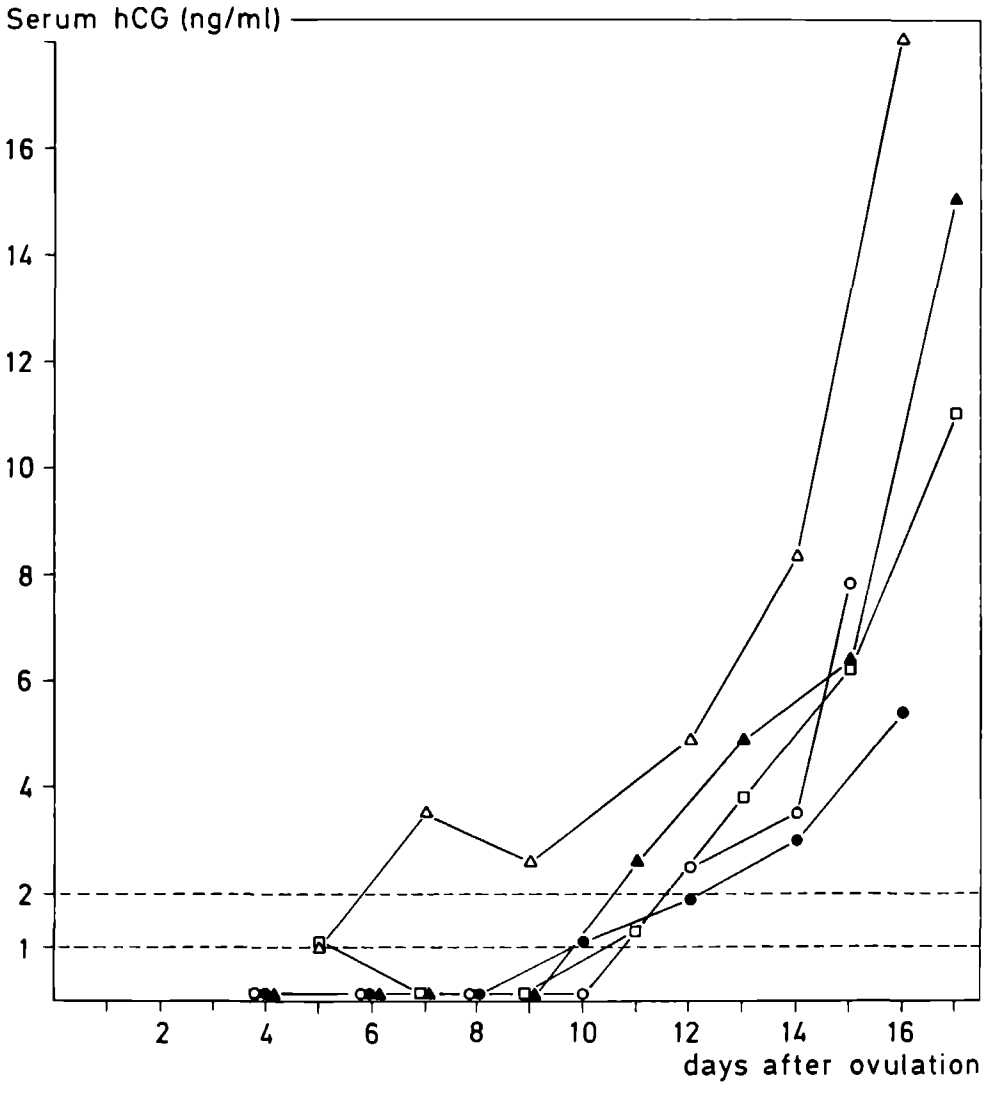


**Figure 5.1** Measurements of Total hCG during the luteal phase of five women with clinical, ongoing pregnancies

**Table V.2** Maximal total hCG, free hCG ß-subunit and intact hCG values in the luteal phase of the menstrual cycle of 20 assumed fertile women and 20 women with unexplained infertility.

| | Infertile (n=20) | Fertile (n=20) |
|---|---|---|
| Total hCG (ng/mL) | | |
| < 1.0 | 17 | 2 |
| 1.0 - 1.9 | 3 | 9 |
| ≥ 2.0 | - | 9* |
| Free hCG ß-subunit (mIU/L) | | |
| < 100 | 15 | 11 |
| ≥ 100 | 5 | 9* |
| Intact hCG (IU/L) | | |
| < 2.5 | 18 | 14 |
| ≥ 2.5 | 2 | 6* |

* 5 clinical pregnancies

### 5.3.2 Free hCG ß-subunit

Only in one woman with unexplained infertility not only elevated total hCG concentrations but also detectable free hCG ß-subunit levels were detected. Furthermore, in four other infertile women free hCG ß-subunit was detected during the luteal phase, however, only on one occasion in each woman.

In the fertile group two of the 13 women with elevated total hCG values without a delay in menstruation, showed elevated free hCG ß-subunit levels on at least one occasion. In 10 women no free hCG ß-subunit was detected despite elevated total hCG levels. In the five women with clinical pregnancies free hCG ß-subunit was detected in the luteal phase, but the first day of detection of total hCG always preceded the first day of detection of free hCG ß-subunit. (table V.3)

In both groups no correlation was found between the actual values of total hCG and free hCG ß-subunit.

**Table V.3** First day of detection of hCG in the luteal phase of five women with clinical, ongoing pregnancies (day 0 = ovulation)

| Patients | First post-ovulatory day of detection of Total hCG | Free hCG ß-subunit | Intact hCG |
|---|---|---|---|
| A-3 | 10 | 16 | 12 |
| A-9 | 12 | 15 | 10 |
| A-14 | 5 | 14 | 12 |
| A-16 | 5 (11)* | 15 | 11 |
| A-20 | 11 | 14 | 9 |

* The day of a second rise of detectable hCG is given between brackets

On one occasion, after an initial rise, hCG decreased below the detection level and showed a second increase which continued.

### 5.3.3 Intact hCG

In two women with unexplained infertility intact hCG was detected during the luteal phase.

In the fertile group intact hCG was detected in five women with clinical pregnancies. The first day of detection of intact hCG is given in table V.3. Furthermore in one presumed fertile woman a concentration of 3.2 IU/L intact hCG was detected on the 11$^{th}$ day after ovulation, despite a normal menstrual cycle. The concentrations of intact hCG of the two infertile women and one fertile woman who had elevated intact hCG levels without a delay of their menstrual periods, were 1/4 to 1/10 of those in the pregnant women on the corresponding days.

## 5.4 DISCUSSION

Evidence for the existence of very early conceptual loss is documented by several methods: morphological, statistical, and biochemical. HCG is a very reliable marker of early pregnancy.[9] Although it is known from *in vitro* studies that the blastocyst itself is capable of producing hCG before implantation,[10,11] it is assumed that hCG appears in maternal blood at the time of implantation, at around 8-9 days after ovulation.[12,13] A rise in plasma hCG reflects the presence of a functional trophoblast and thereby confirms the existence of a probable pregnancy.[14]

Problems exist in the determination of hCG. The early detection of hCG depends on the degree of analytical sensitivity of the assay (i.e. the lowest detectable concentration of hCG), while the specificity of an hCG immunoassay depends to a great extent on the antibodies used, with little cross-reaction of components with structural similarities. An

ideal assay has a high sensitivity, i.e. it is able to quantify very low hCG concentrations, and has also a high specificity, i.e. it does not detect hCG when no hCG is present. The hCG molecule consists of a hormone-nonspecific α-subunit and a hormone-specific ß-subunit, synthesised separately.[15,16] The α-subunit is almost identical to the α-subunits of Luteinising Hormone (LH), Follicle Stimulating Hormone (FSH), and Thyroid Stimulating Hormone (TSH). The amino acid sequence of the hCG-ß subunit bears 80% similarity to that of the LH-ß subunit. Immunochemical assays for the detection of hCG make use of specific antibodies against the ß-subunit or against the intact hCG molecule. The first RIA used in this study applies an antiserum against hCG-ß and is designated a two-component assay, since it detects both the whole hCG molecule and the free hCG-ß subunit ('total hCG').[8] In other reports similar two-component assays were called 'hCG-ß', a name which creates confusion. Furthermore hCG was determined by a specific RIA which detects the free ß-subunit only and an IEMA for the determination of the intact hCG molecule.

An additional problem in the immunochemical measurement of hCG is the determination of biologically inactive but immunoreactive components circulating in the blood, such as the precursors and metabolites of hCG, or degraded hormone fragments.

Furthermore, confusion is brought about by the unstructured use of the three different reference standards, i.e. the 1st IRP-hCG, the 1st IRP-hCG-ß, and the 2nd IS. The use of different hCG assays and different reference standards make it difficult to compare various reports about early hCG detection.

If the lower limit of 1.0 ng hCG/mL would be considered the definition for conception, in this study, three of 20 patients with unexplained infertility (15%) and 13 of 20 presumed fertile women (65%) showed early conceptual loss, whereas the probability of fertilisation in the fertile group was 0.90 (18/20). The studies of Biggers[3] and Roberts and Lowe[2] showed similar data, although based on other methods. However, when the cut-off value of total hCG was considered 2.0 ng/mL, no early conceptual losses were found in the infertile group and four early conceptual losses were seen in the fertile group. This is in agreement with the study of Sharp et al[17], who found that 'apparent' early conceptual loss in non-pregnancy cyles was associated with eventual fertility.

Although the clinical sensitivity of the total hCG RIA is 2.0 ng/mL the difference in the number of women with total hCG levels more than 1.0 ng/mL in the two groups is surprising. This difference cannot be explained by cross reaction of LH. In both groups the LH concentrations were in the normal range of the luteal phase, and no statistically significant differences could be shown between the two groups studied. However, these assumed early conceptual losses could not be confirmed by the assay of free hCG ß-subunit nor by the assay of intact hCG.

The hCG IEMA which detects only intact hCG, has a very high specificity. This assay shows no cross reaction with LH, FSH, or TSH or with the free ß-subunit of hCG. The analytical and clinical sensitivity of this assay are both 2.5 IU/l. Biochemically the determination of intact hCG would be the most indicative method to demonstrate conception. In the five fertile women with clinical, ongoing pregnancies intact hCG was detected before their missed period. In one fertile woman and two infertile women intact

hCG was demonstrated, but in much lower concentrations. Based on the assumption that the determination of intact hCG is the most appropriate method to detect conception at an early stage, one early conceptual loss was seen in the fertile group and two women in the infertile group showed this phenomenon.

Variable quantities of intact hCG and of free subunits have reported to be present in pregnancy sera and the sera of patients with gestational trophoblastic disease.[18-20] The mechanism of regulation of hCG production in early pregnancy is largely unresolved. It has been suggested in an earlier report[21] that a difference might exist between the immunologic and biologic activities of hCG. Further study is required to investigate the relationship between intact hCG, free hCG ß-subunit and other bioactive and/or immunoreactive hCG compounds to explain the apparently conflicting results in the most early stage of pregnancy. Furthermore, heterogeneity of both the circulating α and ß subunits has been demonstrated.[22]

In none of the studies on early conceptual loss intact hCG was determined, and the assumptions of early conceptual loss were based on assays which make use of antibodies against the ß-subunit of hCG and thus detect both the whole hCG molecule and the free ß-subunit.

The first day of detection of hCG in the three different assays of this study did not agree with each other. The assays of 'total hCG' and 'intact hCG' were more sensitive in early detection of hCG than the assay of the free ß-subunit. The range in first day of detection of hCG in the luteal phase in case of clinical pregnancy was high. (fig. 5.1) Since this difference cannot be explained on biological or biochemical grounds, this indicates even more the need for very cautious interpretation of the results of different hCG measurements.

In view of these findings it is highly possible that the frequency of early conceptual loss based on hCG(-ß) determinations only, is overestimated. Early conceptual loss after implantation of the conceptus forms no major explanation for unexplained infertility. The assays of total hCG and intact hCG are useful markers for the detection of the fertilised ovum around the time of implantation. However, these tests cannot detect earlier loss of the ovum. New methods for earlier detection of pregnancy have been developed,[23] and their usefulness in clinically unrecognised early pregnancy loss should be investigated.

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# CHAPTER 6

# ZONA-FREE HAMSTER OVUM SPERM PENETRATION ASSAY

## 6.1 INTRODUCTION

In the investigation of male fertility attention is mainly paid to the classical semen parameters: volume, density, motility and morphology. The conventional semen analysis (SA) gives more information on the quantity of morphologically normal, well moving spermatozoa than on their functional capacity.

Supplementary information on sperm quality can be obtained by the *in vitro* sperm mucus penetration meter (SPM) test according to the method of Kremer.[1] This assay evaluates the capacity of spermatozoa to penetrate into human cervical mucus *in vitro*. *In vivo* the sperm cells migrate into the uterus and Fallopian tubes through active movement to reach the oocyte. The spermatozoa acquire their fertilising capacity during their ascent in the female genital tract.[2] This process is called capacitation. The next essential event for spermatozoa to penetrate the surrounding layers of the ovum is the acrosome reaction. A spermatozoon that has passed through the zona pellucida of the oocyte fuses with the egg plasma membrane, the vitellus, and the sperm nucleus with chromatine decondensates and develops into a sperm pronucleus. The sperm pronucleus then fuses with the egg pronucleus: the actual conception takes place. Until recently it was impossible to determine the fertilising capacity of spermatozoa in the routine fertility work up of the male. With the introduction of the zona pellucida-free hamster ovum sperm penetration assay (SPA), a more direct test for the evaluation of the fertilising capacity of human spermatozoa became available.[3] After washing and preincubation of the ejaculate, the spermatozoa are incubated together with zona-free hamster oocytes and finally the eggs are examined on sperm penetration. A positive SPA means that the sperm cells are capable to capacitate, to undergo the acrosome reaction, to enter and fuse with the vitelline membrane and to undergo the initial stages of nuclear decondensation.[4,5] However, a positive result of the SPA does not always mean that the investigated male is fertile.[6] In order to achieve conception *in vivo* the spermatozoa must traverse the cervical mucus and forward motility is required to ascend in the female genital tract to reach the site of fertilisation. Furthermore the spermatozoon has to pass the zona pellucida. This function of the sperm cells is not determined in the SPA, for the surroundings of the ova are removed from the hamster oocytes. On the other hand, a negative SPA does not necessarily reflect the inability of spermatozoa to fertilise human oocytes *in vivo*.[7-9]

Since the introduction of the SPA, over 300 publications have appeared on this subject. The widespread use of the test has created controversies about the usefulness and value of the SPA as an additional parameter of the fertility investigation. The

outcome of the SPA is influenced by many variables. A crucial factor in the interpretation of the result is the viability and the motility of the spermatozoa after the *in vitro* capacitation process.[10]

In this investigation the correlation between motility after preincubation and the outcome of the SPA has been studied. Furthermore the influence of two different methods of sperm washing on the motility after preincubation and on SPA results are considered. The correlations between SPA and routine semen parameters, between SPA and SPM-test and between SPA and IVF results are reported and discussed.

## 6.2 CORRELATION BETWEEN THE ZONA-FREE HAMSTER OVUM SPERM PENETRATION ASSAY AND THE CONVENTIONAL SEMEN ANALYSIS PARAMETERS

### 6.2.1 Introduction

Routine SA is the most conventional method to obtain knowledge on male fertility. In 1677 Antoni van Leeuwenhoek was probably the first person to observe spermatozoa in the human ejaculate.[11] During especially the last three decades research on semen has made a remarkable progress and the SA has become routine in the male fertility investigation. The main parameters of the SA are density, motility, and morphology. The conventional SA proceeds from the assumption that conception *in vivo* will result if there is a certain critical number of motile and morphologically normal spermatozoa in the ejaculate. Routine SA gives information on the quantity rather than on the quality of the semen. The SPA has been advocated as a qualitative assay for the evaluation of the fertilising ability of human spermatozoa. A survey of the literature of the SPA shows controversies about the correlations between the SA and the SPA.(see for review 4,5,12) In order to determine whether the presence of impaired penetrating capacity can be predicted from the routine SA, the conventional semen parameters were considered versus the SPA results.

### 6.2.2 Materials and methods

#### *6.2.2.1 Patients*

The study group consisted of 220 males of infertile couples. The men were not further divided into infertile, subfertile and potentially fertile, because infertility frequently cannot be assigned with certainty to either partner of an infertile couple. In all couples the involuntary infertility existed for at least one year. Only one semen sample from each man was used for the study.

#### *6.2.2.2 Semen analysis*

After at least 48 hours of sexual abstinence semen samples were produced by masturbation and collected in a sterile container. The samples were allowed to liquefy

for 30 to 60 minutes at room temperature. The ejaculates were analysed within two hours after collection. Each semen sample was examined with the use of conventional techniques for determinination of semen volume, sperm density, percentage of motile spermatozoa, motility grade and percentage of morphologically normal spermatozoa. The grade of motility was expressed in a motility score: 1: immobile, 2: poor, 3: moderate, 4: fairly good, 5: good, 6: excellent.

Semen characteristics were considered to be normal with a sperm density of at least 20 x $10^6$/mL, at least 50% motile spermatozoa (two hours after collection), motility grade at least 4, and 40% or less morphologically abnormal spermatozoa. If one or more of these parameters were outside the normal range, the semen sample was considered to be abnormal.

Screening on sperm antibodies was performed by the Mixed-Antiglobulin-Reaction (MAR) test which makes use of antihuman IgA and IgG antibodies linked to polyacryl amide beads (Bio Rad Laboratories BV, Utrecht, The Netherlands).[13] None of the samples included in this study showed evidence of agglutination or a positive MAR-test.

*6.2.2.3 Zona-free hamster ovum sperm penetration assay*

A part of the liquefied semen sample was diluted with Biggers-Whitten-Whittingham (BWW) medium[14] to 10 mL and centrifuged at 300 g for 5 min. The supernatant was removed and afterwards the pellet was washed twice again with BWW. The last pellet was resuspended in 0.5 to 2.0 mL BWW and adjusted to a final spermatozoal concentration of 5 to 10 x $10^6$/mL and transferred to Falcon tubes (Falcon Plastics 2063, Becton Dickinson Labware, Oxnard, Ca 93030). The tubes were placedhorizontally in an air incubator at 37°C for 18 to 20 hours in order to effect capacitation.

The oocytes were collected from female golden hamsters (Mesocricetus auratus), which were induced to superovulate with human menopausal gonadotrophin (HMG, Humegon®, Organon, Oss, The Netherlands) and human chorionic gonadotrophin (hCG, Pregnyl®, Organon, Oss, The Netherlands). The corona radiata was dispersed by bringing the oocytes in a hyaluronidase solution (Hyason®, 150 U/mL, Organon, Oss, The Netherlands). The zona pellucida was removed with 0.1% bovine pancreatic trypsin (type III, Sigma Chemical Co). After washing of the oocytes with BWW (3 mg/mL serum albumin) three times, the zona-free hamster oocytes were placed in Falcon petri dishes (Falcon Plastics 3001, Becton Dickinson Labware, Oxnard, Ca 93030) containing 0.3 mL BWW (30 mg/mL serum albumin) and covered with paraffin. Unlike other studies the SPAs were performed with approximately ten hamster oocytes. In an earlier, not yet published, experiment of 235 SPAs, it was proven that the penetration rate of SPAs calculated on 10 ova is not statistically different from the penetration rate of SPAs calculated on all available oocytes, varying from 11 to 30 (p=0.96). Also, the percentage of SPAs with at least one penetrated ovum was 52.7% when only the first 10 ova were examined, whereas when all the ova were assessed this percentage was 60.6% (not significant).

The spermatozoal suspension was added to the hamster ova and after an incubation period of two to three hours (37°C) the eggs were examined on sperm penetration. The presence of a swelling sperm head or pronuclei connected to a visible sperm tail in the cytoplasm was considered a positive assay for penetration. The penetration rate (in %) was defined as the number of penetrated oocytes divided by the total number of oocytes multiplied by 100. Penetration of at least one egg was considered a positive SPA. A negative SPA was defined as a penetration rate of 0%.

*6.2.2.4 Statistics*

The statistical comparison between percentages for two different groups was performed using the chi-square test for the 2 x 2 table. The contingency coefficient was used as a measure for the strength of the dependence between two dichotomous variables.

### 6.2.3 Results

A positive SPA was found in 149 of the 220 samples (68%). A completely normal SA was determined in the samples of 104 men, of whom 86% had a positive SPA. The remaining 116 males had one or more abnormal semen characteristics, and 52% of this group had a positive SPA.

Of the 220 semen samples 195 had a normal density and 25 had a density of less than 20 x $10^6$/mL. Less than 50% motile sperm cells were seen in 32 samples; 188 had at least 50% motile spermatozoa. The grade of motility was moderate or less in 24 samples and fairly good, good or excellent in 196 cases. In 117 ejaculates morphological examination showed no more than 40% abnormal forms and in 103 semen samples more than 40% abnormal spermatozoa were seen. For each semen parameter the number and frequency of positive SPA scores are shown in table VI.1. The differences between the number of positive SPAs for a 'good' or for a 'not-good' semen parameter were always statistically significant.

**Table VI.1** Frequency of positive SPAs in relation to the semen characteristics (n=220)

| Semen characteristic | Category 'not-good' | Percentage pos. SPA | Category 'good' | Percentage pos. SPA |
|---|---|---|---|---|
| Density | <20 x $10^6$/mL | | ≥20 x $10^6$/mL | |
| | 6/25 | 24% | 143/195 | 73% |
| % Motility | <50% | | ≥50% | |
| | 13/32 | 41% | 136/188 | 72% |
| Motility grade | <4 | | ≥4 | |
| | 9/24 | 38% | 140/196 | 71% |
| Abnormal forms | >40% | | ≤40% | |
| | 51/103 | 50% | 98/117 | 84% |

In the same way the mean penetration rates and the standard deviations in relation to the different semen characteristics are shown in table VI.2. The large standard deviations reflect the extensive variability in penetration rate over the different samples. It was also found that the more normal semen characteristics in the semen analysis were present, the more a positive SPA was seen. In the total group of 220 men 15 had 4 abnormal semen parameters. Only 2 men in this group had a positive SPA.

**Table VI.2** The mean penetration rates and standard deviations of 220 SPAs in relation to the semen characteristics

| Semen characteristic | Category 'not-good' | Penetration rate (%±SD) | Category 'good' | Penetration rate (%±SD) |
|---|---|---|---|---|
| Density | $<20x10^6$/mL | 10 ± 25% | $\geq 20x10^6$/mL | 38 ± 36% |
| % Motility | <50% | 21 ± 14% | ≥50% | 37 ± 36% |
| Motility grade | <4 | 17 ± 29% | ≥4 | 37 ± 37% |
| Abnormal forms | >40% | 21 ± 30% | ≤40% | 47 ± 37% |

Up to now there is no agreement on the definition of a positive SPA. The limits mostly applied to define a positive SPA are a penetration rate of more than 0% or more than 15%. To determine the most appropriate definition of a positive SPA the contingency coefficients between the different semen parameters and the SPA for two different dichotomisations, '0%' and '15%', respectively, have been assessed. (table VI.3) The contingency coefficient is used to express the strength of the relationship. It shows that all semen characteristics have the strongest correlation with the result of the SPA with a lower limit of positive SPA as more than '0%'in comparison to a limit of '15%'.

**Table VI.3** Contingency coefficients between the different semen characteristics and the SPA for two definitions of positive SPA

| | DEFINITION OF POSITIVE SPA: | |
|---|---|---|
| Semen characteristic | Penetration rate>0% | Penetration rate>15% |
| Density | 0.318 | 0.224 |
| Motility percentage | 0.233 | 0.176 |
| Motility grade | 0.221 | 0.129 |
| Morphology | 0.343 | 0.291 |

### 6.2.4 Discussion

The routine SA gives insufficient information on the actual function of the spermatozoa. The SPA has been advocated as a more direct test to assess the *in vitro* fertilising ability of human spermatozoa.

To determine whether the SPA gives additional information in the fertility investigation of the male, the relation between the routine SA and the SPA is studied in a group of 220 men of infertile couples. Furthermore, the strength of the correlation between the outcome of the SPA and the classical semen characteristics for two different definitions of a positive SPA has been assessed. Allsemen parameters showed the best correlation with the SPA with a lower limit of penetration of 1%. For that reason and because of the smaller number of ova used per assay (usually approximately ten) in comparison to other investigators, any sample with penetration at any level between 1% and 100% has been considered as a positive SPA. Several investigators have published on the range of penetration rates seen in 'fertile' and 'infertile' populations. The lower range of penetration for the fertile men varies between 0 and 25%.[6,8,9,15-22] The margin of difference between infertile and fertile men is not clear.

The study group consists of infertile, subfertile and fertile men. It can be expected that in this group a large variation in SA values is seen. To detect a possible correlation between parameters of the SA and the SPA, a large variation in SA data is required and the group has not been divided into (sub)fertile and infertile subjects.

It has been shown that the ability to penetrate hamster oocytes depends on whether or not the routine semen characteristics meet the criteria for normal semen analysis values (table VI.2). Every normal semen parameter showed a statistically significantly higher chance to contribute to a positive SPA compared with an abnormal semen parameter. Furthermore, the more normal semen parameters in the SA were present, the more males showed a positive SPA. In the study group as a whole a significant correlation was demonstrated between the SA and the SPA, but the large variation in SPA results reflect the inability to predict the SPA outcome of an individual case. In the presence of a normal SA, the SPA was most often positive and in case of a severely impaired SA the SPA was negative in the majority of men. When only one semen characteristic was 'not good' the SPA result could not be predicted.

The potential of the SPA in screening defects of sperm function which are normally not detected by routine SA, has been evaluated by a number of investigators. Unfortunately, it appears that there is no concensus on the predictive value of the test.[6,8,15-19] Most investigators could not demonstrate a dependence of sperm penetration rates upon sperm concentration, motility and morphology.[8,9,15-17] Cohen et al.[18] observed a low but significant correlation between the percentage of normal spermatozoa and the SPA. Rogers et al.[19] also showed that the SPA correlated best with sperm morphology. Wickings et al.[23] found no difference in penetration rates between infertile men with normal and with abnormal seminal parameters. The degree of correlation reported may depend on the selection of the group of samples.

Reduced penetration rates can be a consequence of the functional incompetence of the spermatozoa or can be due to reduced normal, motile sperm numbers.[24] In case of a negative SPA and an abnormal SA it is not possible to determine whether the spermatozoa failed to penetrate due to an intrinsic defect of sperm function or due to a reduced sperm number.

## 6.3 IMPORTANCE OF SPERM MOTILITY AFTER PRE-INCUBATION IN THE INTERPRETATION OF THE ZONA-FREE HAMSTER OVUM SPERM PENETRATION ASSAY

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### 6.3.1 Introduction

There is no standardised procedure for the performance of the SPA. The outcome of the SPA is dependent on many variables in the technical performance of the test.[5] The preincubation time of sperm is an extremely important variable in the procedure.[25] During the preincubation period the spermatozoa have to undergo capacitation and acrosome reaction as mentioned previously.

A crucial factor in the interpretation of the outcome of the SPA is the survival and motility of the spermatozoa after the *in vitro* capacitation process, just before they are added to the hamster oocytes.

The study in this chapter evaluates the influence of the motility after preincubation on the results of the SPA for both normal and abnormal semen samples.

### 6.3.2 Material and methods

The study group consisted of 220 men as described previously (6.2.2.1). SA and SPA were carried out as described in 6.2.2.2 and 6.2.2.3, respectively. Additionally the motility grade after the preincubation period of the spermatozoa was determined in the same way as the motility grade was scored in the routine SA.

### 6.3.3 Results

After the preincubation period of 18 to 20 hours the spermatozoa of 69 semen samples were immobile or moved poorly or moderately. A motility grade of 4 or more was seen in 151 ejeculates after washing and preincubation. Initially 196 males had a motility grade of 4 or more (Table VI.4). In 53 of these the motility was impaired to a grade 3 or less after washing and preincubation. Of the 24 semen samples with an insufficient initial motility 8 improved after washing or during the preincubation period. In 159 cases the motility grade remained unchanged.

**Table VI.4** Influence of washing and preincubation on the motility grade

| Motility grade | | After preincubation | |
|---|---|---|---|
| | | <4 | ≥4 |
| Before preincubation | <4 | 16 | 8 |
| | ≥4 | 53 | 143 |

When the motility after capacitation *in vitro* was compared with the results in the SPA, for both the samples with an abnormal and those with a normal spermiogram, statistically significant differences in positive SPAs were found between samples with a normal and with an abnormal motility after preincubation (table VI.5).

**Table VI.5** Frequency of positive SPAs in relation to the motility afterpreincubation for 220 semen samples with a normal or abnormal routine semen analysis

| Motility grade after preincubation | SA abnormal | | SA normal | |
|---|---|---|---|---|
| Immotile-Moderate (<4) | 18/54* | 33% | 7/15** | 47% |
| Fairly good-Excellent (≥4) | 42/62* | 67% | 82/89** | 92% |

* $p = 0.001$
** $p = 0.003$

The mean penetration rates and the standard deviations for the semen samples of the four groups are shown in table VI.6. The highest penetration rates were found when the motility grade after preincubation was ≥4.

Of the 220 males, 89 had a completely normal routine SA and a sufficient motility after preincubation. Of these 89 men only 7 had a negative SPA score.

**Table VI.6** Mean penetration rate (% ± SD) in relation to the motility after preincubation and the semen analysis for 220 different semen samples

| Motility grade after preincubation | SA abnormal | SA normal |
|---|---|---|
| Immotile-Moderate (<4) | 10.8 ± 22.6 | 26.7 ± 36.8 |
| Fairly good-Excellent (≥4) | 33.5 ± 33.9 | 51.7 ± 36.3 |

The strength of the relation between the classical semen parameters and the motility grade after preincubation (expressed by the contingency coefficient) is given in table VI.7.

**Table VI.7** Contingency coefficients of the semen parameters of 220 males in relation to the motility grade after preincubation

| Semen characteristic | Contingency coefficient of the motility grade after preincubation |
|---|---|
| Density | 0.326 |
| % Motility | 0.216 |
| Motility grade (SA) | 0.257 |
| Morphology | 0.328 |

Density and morphology showed the highest correlation with the motility grade after preincubation.

The relation between the motility grade after preincubation and the result of the SPA for two definitions of positive SPA (more than 0% and more than 15%) is expressed by the contingency coefficients. The highest contingency coefficient was found when the lower limit of a positive SPA was 1% (contingency coefficient 0.414) in comparison to a lower limit of 16% (contingency coefficient 0.376). Both for the definition 'more than 0%' and 'more than 15%', the motility grade after preincubation showed the strongest correlation with the SPA score in comparison to the classical semen characteristics.(Table VI.3)

### 6.3.4 Discussion

The outcome of the SPA is influenced by the protocol used to perform the assay. One of the most important of these is the preincubation period. During this period the capacitation and acrosome reaction, both essential conditions for penetration of the oocyte, must occur.[4,12] During the washing procedure and/or the preincubation period the motility of the spermatozoa can change.

This study showed a statistically significant decrease in the number of positive SPA scores when the motility grade of the spermatozoa after the preincubation period was <4, compared with semen samples with a motility of ≥4 after preincubation. Motility after preincubation showed a stronger correlation with the SPA than any of the other classical semen characteristics.

There is no concensus about the ideal preincubation period, possibly because the time required for capacitation is different for each individual.[5,12] The preincubation period of the 220 samples of this study was 18 to 20 hours, to provide every ejaculate ample time to capacitate. Some authors have reported better results in the SPA after an incubation

time of 19 to 20 hours, compared with a shorter period of 6 to 7 hours.[25,26] In another study, no difference in penetration rate was noted when sperm was left to capacitate either for 5 to 7 hours or for 18 to 20 hours before insemination.[23] In contrast, Zausner-Guelman et al.[17] reported a nearly opposite result: sperm preincubated for 2 to 3 hours produced higher penetration levels than did those preincubated for 17 to 24 hours. In the same study a positive correlation existed between sperm survival at the end of the preincubation period and the SPA score. This is in agreement with our study.

Some studies have indicated that the outcome of the SPA is influenced by the concentration of motile spermatozoa in the incubation medium.[27,28] Aitken et al.[29] demonstrated in a group with unexplained infertility that 91% of the positive SPAs were correctly identified by the postcapacitation movement characteristics. Cohen et al.[18] could not demonstrate a significant correlation between the motility of the sperm cells at the end of the preincubation period and fertilisation percentage of the hamster oocytes. Gould et al.[30] observed a decline in sperm motility during preincubation, but this had no influence on the result in the SPA. To avoid possible false-negative SPAs due to insufficient density of motile spermatozoa, it has been suggested to standardise the SPA for a fixed concentration of motile spermatozoa.[31]

From this study it can be concluded that insufficient motility after preincubation of the spermatozoa is an important factor determining the result of the SPA. The motility after preincubation must be taken into account when the SPA score is negative.

## 6.4 THE INFLUENCE OF WHITE BLOOD CELLS AND PERCOLL WASHING ON THE ZONA-FREE HAMSTER OVUM SPERM PENETRATION ASSAY

### 6.4.1 Introduction

Surprisingly, the seminal fluid is supposed to be a sub-optimal environment for spermatozoa, especially when the semen plasma contains white blood cells.[32] Recently it has been reported that the presence of inflammatory cells in the semen impairs the penetrating ability of spermatozoa both in human oocytes and in zona-free hamster oocytes.[33,34]

Various methods are described to separate spermatozoa from other components in the semen.[35-38] Gorus and Pipeleers[39] first introduced the use of Percoll to isolate human spermatozoa from semen and other seminal components and to fractionate these spermatozoa according to their relative motility. It has been reported that the yield of spermatozoa with this method is at least as good as any other separation technique.[40] The Percoll centrifugation method has advantages in terms of recovery rate.[41] Forster et al.[42] examined the influence of the Percoll separation procedure on sperm motility, morphology and sperm function using the zona free hamster ova sperm penetration assay (SPA). They found an enhanced SPA-score after Percoll centrifugation in comparison with the conventional sperm washing method with modified BWW.

The aim of this study was to investigate the influence of Percoll layer centrifugation on the survival rate after overnight preincubation and the hamster ova penetration rate of semen samples with and without white blood cells (WBCs).

### 6.4.2 Materials and methods

The study group consisted of 291 men of infertile couples. Only one semen sample of each man was admitted to the study.

The number of WBCs was counted in the Makler counting chamber with the use of phase contrast microscopy (magnification 100x) for discrimination between WBCs and immature germ cells. The upper limit for WBCs in a normal semen sample is $1x10^6$/mL.

Two methods were used for sperm preparation: the first method as described in 6.2.2.3, the BWW procedure, and by the second method the sample was centrifuged on a 80% Percoll layer after liquefaction and than washed with BWW.

### 6.4.3 Results

No white blood cells were seen in 202 semen samples; 151 of these were washed with modified BWW and 51 with Percoll. Of the 89 semen samples which contained white blood cells, 62 were washed with modified BWW and 27 with Percoll. WBCs were never seen in the specimen after centrifugation of the semen on a 80% Percoll layer.

The presence of white blood cells in the ejaculate had a negative influence both on the percentage of men with a positive SPA-score and on the penetration rate (table VI.8, $p<0.05$).

No statistically significant differences were seen between the BWW washing procedure and the Percoll centrifugation, either in the percentage of men with a positive SPA, nor in the penetration rate. This observation was independent of the presence of leucocytes in the ejaculate before centrifugation.

**Table VI.8** Percentage of semen samples with a positive SPA-score and mean penetration rate (%Pen) related to the presence of white blood cells (WBCs) before centrifugation and the method of semen washing

| | Modified BWW | | | Percoll | | |
|---|---|---|---|---|---|---|
| | Positive SPA | | %Pen | Positive SPA | | %Pen |
| WBCs absent | 72% | (109/151) | 40 | 61% | (31/51) | 30 |
| WBCs present | 58% | (36/62) | 21 | 52% | (14/27) | 25 |

The mean penetration rate of semen samples containing white blood cells when centrifuged on a Percoll layer was not different from semen samples washed with BWW.

A strong correlation exists between the motility after preincubation and the SPA result.(see 6.3). The motility after preincubation in relation to the presence of WBCs is given in table VI.9. Both in the absence as well as in the presence of white blood cells, modified BWW-washing gave a slightly better motility after preincubation than the Percoll-method (Table VI.9).

**Table VI.9** Percentage of semen samples with motility after preincubation ≥4 related to the presence of white blood cells (WBCs) before centrifugation and the method of semen washing

| | Modified BWW | | Percoll | |
|---|---|---|---|---|
| WBCs absent | 71% | (107/151) | 65% | (33/51) |
| WBCs present | 66% | (41/62) | 59% | (16/27) |

In the second part of the study, ejaculates of 12 men were collected. Half of the ejaculate was submitted to the modified BWW washing method and the other half to the Percoll centrifugation method (table VI.10).

In 5 ejaculates a minimum of 1 x $10^6$/mL leucocytes were present; 3 had a negative SPA-score both after the modified BWW and the Percoll method and 2 had a positive score. These samples showed a slightly higher penetration rate after Percoll washing.

Of the 8 semen samples without leucocytes, 2 had a negative SPA both after BWW and Percoll; 4 showed a slight but not significant increase of the penetration rate after Percoll washing. In one semen sample the SPA was negative after BWW washing and positive after Percoll centrifugation (penetration rate 33%). In contrast, in one ejaculate the penetration rate decreased from 100% to 31% after Percoll washing, despite a very good motility after the preincubation period.

**Table VI.10** Routine semen characteristics, motility after preincubation and penetration rate of 13 semen samples which partially were submitted to the modified BWW washing method and partially to the Percoll centrifugation method

| Pat nr | Vol mL | Count x10$^6$/ mL | Leuco x10$^6$/ mL | %Mot | Mot Grade | Abn Forms % | Mot after preinc BWW | | Percoll | | %Penetration BWW | Percoll |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 220 | 5.7 | 22 | - | 50% | 6 | 49 | 30% | 4 | 10% | 2-3 | 0% | 0% |
| 225 | 4.4 | 20 | 3 | 100% | 5 | 45 | 100% | 2 | 10% | 5 | 0% | 0% |
| 226 | 3.3 | 77 | 5 | 100% | 4-5 | 50 | 40% | 5 | 100% | 6 | 29% | 33% |
| 234 | 5.3 | 32 | 6 | 30% | 3-4 | 47 | 10% | 5 | | 1 | 0% | 0% |
| 235 | 4.5 | 49 | - | 100% | 6 | 40 | 100% | 6 | 100% | 6 | 30% | 50% |
| 247 | 3.1 | 60 | - | 100% | 5 | 35 | 100% | 5 | 70% | 6 | 0% | 10% |
| 254 | 6.0 | 54 | - | 100% | 5 | 32 | 100% | 6 | 100% | 6 | 55% | 70% |
| 258 | 3.4 | 64 | - | 100% | 5 | 44 | 10% | 4 | 30% | 5 | 27% | 44% |
| 259 | 4.0 | 135 | - | 100% | 5 | 25 | 100% | 6 | 100% | 6 | 100% | 31% |
| 285 | 2.5 | 13 | 1 | 100% | 4 | 37 | 10% | 2 | 10% | 2 | 0% | 0% |
| 288 | 1.2 | 40 | 1 | 20% | 4 | 62 | 10% | 2 | 10% | 3 | 20% | 30% |
| 295 | 3.4 | 75 | - | 50% | 5 | 60 | 10% | 2 | 50% | 5 | 0% | 0% |
| 302 | 5.7 | 61 | - | 30% | 4 | 46 | 30% | 3 | 40% | 5 | 0% | 33% |

### 6.4.4 Discussion

Previous studies have shown that seminal plasma affects the fertilising capacity of spermatozoa, probably because semen plasma contains decapacitation factors.[32] *In vivo* the separation of spermatozoa from seminal plasma is one of the most important functions of cervical mucus, and cervical mucus may act as a bacteriological filter.[43] There is no consensus about the method of this separation *in vitro*.[44]

This study examined the effect of two different sperm washing procedures on the ability of human spermatozoa to fertilise zona-free hamster ova, with special emphasis on the presence or absence of white blood cells in the ejaculate. We confirmed previous reports that the presence of white blood cells in semen has a negative effect on sperm function.[33,45-47] The results in the SPA for samples with at least 1 x 10$^6$ WBCs/mL were significantly lower than without WBCs.

In spite of the removal of the leucocytes the semen samples washed with Percoll had less frequently a positive SPA than the samples washed with modified BWW. Furthermore, Percoll did not improve the motility grade after the preincubation period at the time of combination with the hamster oocytes, either in the absence nor in the presence of WBCs in the ejaculate. This is in in agreement with the investigation of Berger et al.[41] who found that the velocity of sperm was not significantly enhanced after the Percoll separation technique, but the ability to penetrate zona-free hamster ova was increased in this study. In contrast, the study of Pousette et al.,[48] reported that in a group of fertile men the Percoll separation technique improved the progressive motility

index. However, when samples from patients with abnormal semen profiles were separated in this way, the degree of improvement was much more variable.

In order to examine the effect of Percoll washing both on motility after preincubation and on the results in the SPA, 13 ejaculates of different men were partially submitted to the modified BWW washing procedure and to the Percoll centrifugation method. Only in two semen sample the penetration rate increased from a negative result to positive with the Percoll procedure. In the other cases a negative SPA score remained negative and a positive result remained positive.

Although Percoll centrifugation can remove the WBCs from the semen, our results indicate that the white blood cells have had their deterious effect on the spermatozoa already. This is in agreement with the study of Rogers et al.,[49] who demonstrated that the effect of seminal plasma on spermatozoa could not be reversed by subsequent washing. Prolonged exposure of the spermatozoa to seminal plasma gave a reduction of fertilisation.

Our study showed no advantage of the Percoll washing procedure on the SPA results and the motility after preincubation.

## 6.5 COMPARISON OF THE ZONA-FREE HAMSTER OVUM SPERM PENETRATION ASSAY AND THE SPERM PENETRATION METER TEST ACCORDING TO KREMER

### 6.5.1 Introduction

An essential part of the investigation of an infertile couple is the evaluation of the interaction between spermatozoa and cervical mucus. The only *in vivo* test for this purpose is the postcoital test or Sims-Huhner test. *In vitro* the penetration and migration of spermatozoa into cervical mucus can be investigated by the sperm penetration meter (SPM) test according to Kremer.[1] The performance of spermatozoa in such a test depends on functions of the sperm cells as well as on the properties of the mucus. A great advantage of the SPM-test above the postcoital test is the possibility to carry out crossed SPM-tests to determine whether the cause of a negative homologous SPM-test is determined by the semen or the cervical mucus. The SPM-test produces objective data about the semen sample: it is also a functional test in which spermatozoa demonstrate their ability to migrate into cervical mucus and to survive.

The SPA measures the ability of spermatozoa to penetrate the oocyte membranes. In this chapter these two functional tests to determine sperm quality are compared.

### 6.5.2 Materials and methods

The SPM-test and the SPA were performed in 140 different semen samples of different men of infertile couples visiting the outpatient department for Gynaecologic Endocrinology and Infertility.

The SPM-test was carried out with human preovulatory cervical mucus of macroscopically good quality. A round glass capillary tube was filled with cervical mucus and placed in a reservoir containing the patient's semen. At a magnification of 100 x the number of spermatozoa per visual field at a certain distance and at a certain time were recorded. The results of the SPM-test were judged and scored with the use of the SPM score according to Roumen,[50] a simplification of the original scoring system of Kremer. (table VI.11)

The SPA was performed as described in 6.2.2.3.

**Table VI.11** The SPM-score according to Roumen

| Judgement | Score | | Minimal no of sperm | On distance (cm) | After time (hrs) |
|---|---|---|---|---|---|
| Good | 5 | | 1 | 5 | 1/2 |
| | | or: | 10 | 5 | 2 |
| Sufficient | 4 | | 1 | 5 | 2 |
| Moderate | 3 | | 1 | 3 | 2 |
| Bad | 2 | | 1 | <3 | 2 |
| Negative | 1 | | 0 | <3 | 2 |

### 6.5.3 Results

The score of the SPM-test was compared with the chance of a positive SPA-score and with the penetration rate in the SPA. Both correlations are presented in figures 6.1 and 6.2.

Of the 140 samples included in this study, 23 had a SPM-score of 2, 20 a SPM-score of 3, 34 had a SPM-score of 4 and there were 63samples with a SPM-score of 5.

Out of the 23 samples with a SPM-score of 2, 6 penetrated at least one egg in the SPA giving a chance of a positive SPA of 0.26; of the 20 samples with a SPM-score of 3 again 6 had a positive SPA; of the 34 samples which scored moderately well (4) in the SPM, 25 scored positive in the SPA and 48 of the 63 samples with a SPM score of 5 had a positive SPA. Figure 6.1 depicts the relation between the SPM-score and the frequency of semen samples which penetrated at least one hamster oocyte in the SPA ($p=0.005$).

In figure 6.2 the significant correlation between the SPM-score and the percentage of eggs penetrated in the SPA is shown. However, in each of the four groups of SPM-scores the range of penetration rates was 0 - 100%.

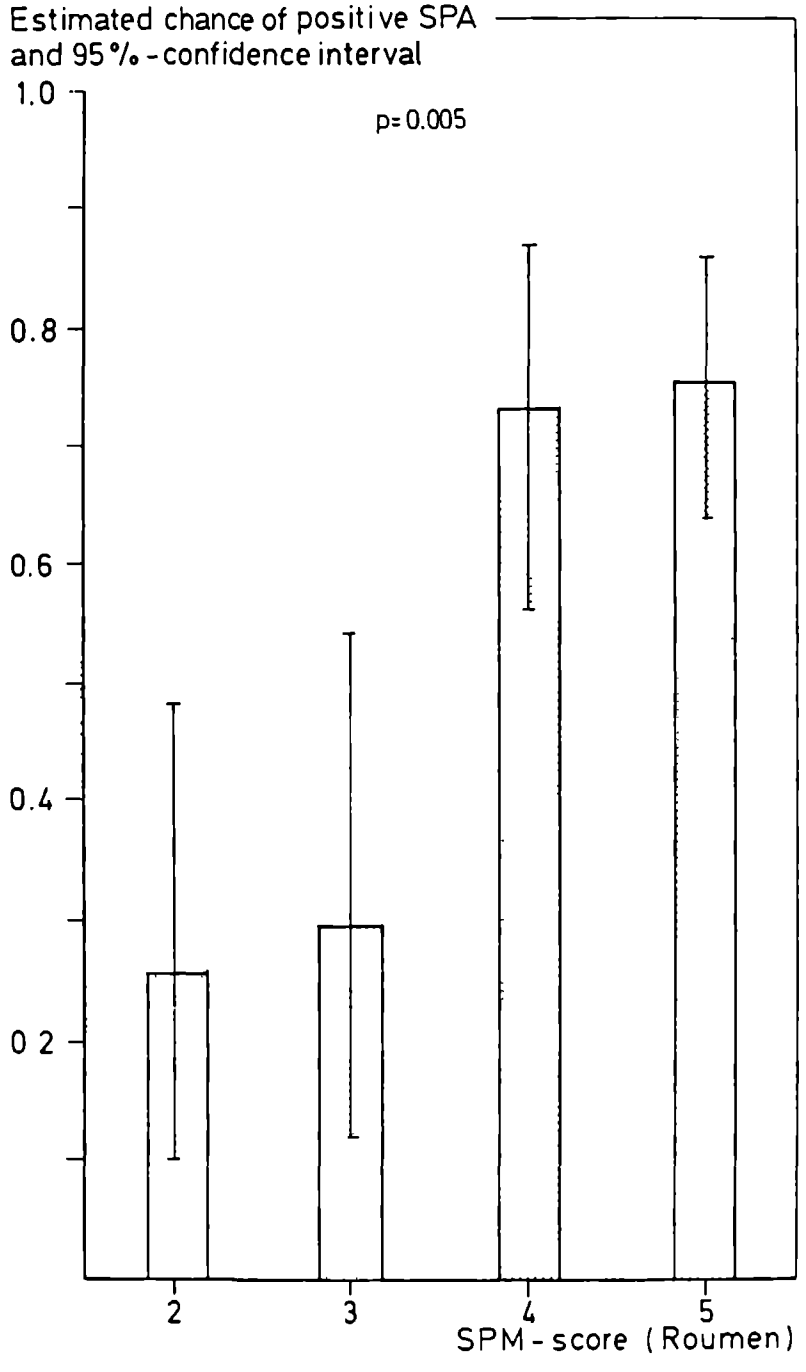


**Figure 6.1** Relation between the SPM score according to Roumen and the frequency of males with a positive SPA for 140 different semen samples

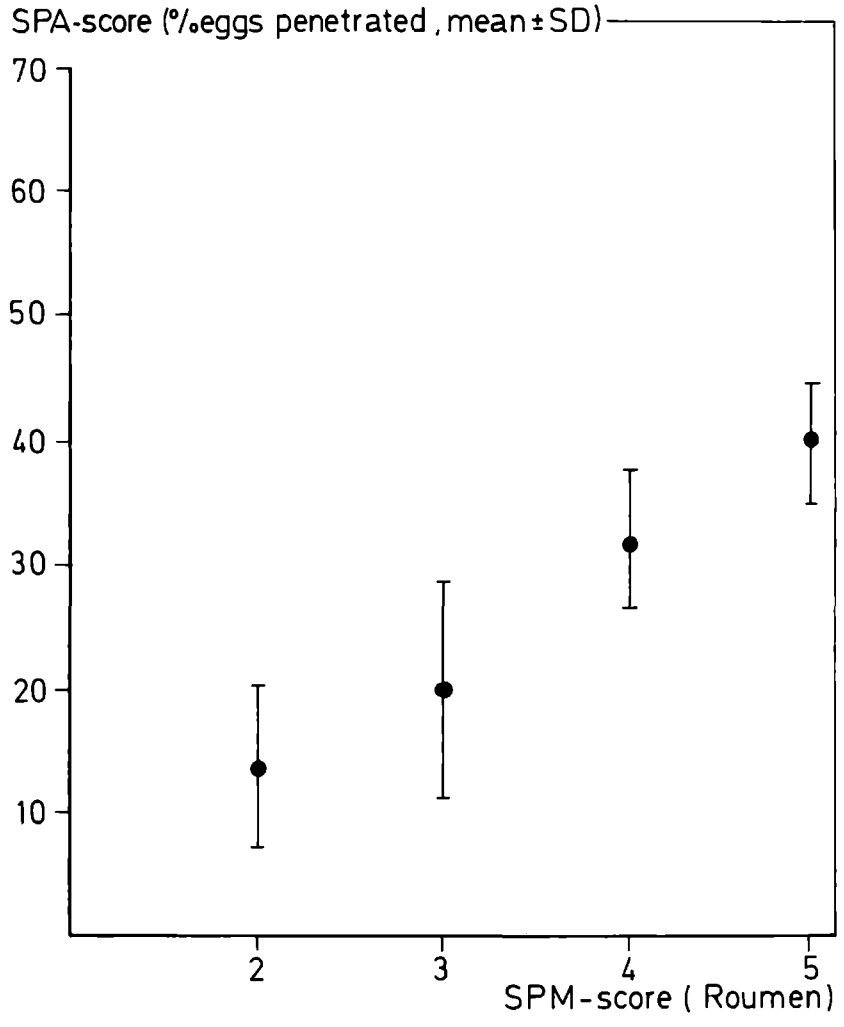


**Figure 6.2** Relation between the SPM score according to Roumen and the penetration rate in the SPA for 140 different semen samples (Mean ± SD)

### 6.5.4 Discussion

The SPM-test and the SPA are qualitative tests which contribute in diagnosing the fertility potential of men. Both laboratory tests are objective assays to simulate processes occuring *in vivo*, namely migration of human spermatozoa into cervical mucus and penetration of the oocyte membrane.

The SPM-test according to Kremer depends on the quality of both the cervical mucus and the sperm cells. The subject of this study was the function of the spermatozoa, therefore the quality of the cervical mucus was standardised.

Positive correlations were observed between the SPM-score and both the percentage of men with a positive SPA and the penetration rate in the SPA. Men with a SPM-score of 4 or 5 (sufficient or good) had significantly more often a positive SPA in comparison to men with a SPM score lower than 4. The penetration rate showed a gradual increase from 14.0% at a SPM-score of 2 to 39.9% when the SPM-score was good (=5). But in all the 4 groups penetration rates ranged from 0% (negative) to 100%. The outcome of an individual SPA could not be predicted accurately by the SPM-test of the same person. This conclusion is also drawn from the study of Takemoto et al.,[51] which suggests that the properties of the sperm cells critical to the two types of penetration are different. Hirschel et al.[52] compared in a group of 30 patients the CMPT (a modified SPM-test) and the SPA and found no correlation between these tests, suggesting that the CMPT and the SPA detect variables which contribute independently to infertility.

A significant relationship between the PCT and the SPA was found in a study of Soules et al.,[53] but the range of the individual values was too large to recommend exclusion of either test as part of a complete infertility evaluation. Controversely, other investigators[54] found a good correlation between the SPA and the ability of spermatozoa to penetrate cervical mucus ($r=0.623$, $p<0.01$). It is suggested that spermatozoa unable to penetrate preovulatory cervical mucus were usually also unable to fertilise the human oocyte.[55]

The common factor influencing cervical mucus penetration and hamster egg penetration may be the specific motility patterns of the spermatozoa. Motility is a critical factor determining both the fertilising capacity of the spermatozoa[10,28] and their ability to migrate through cervical mucus.[35] The SPM evaluates indirectly the motility of the spermatozoa. A negative SPM may reflect a defective sperm function which can also manifest itself in a negative SPA. This study indicates that both assays measure different sperm properties, and that both tests contribute separately to the total infertility investigation.

## 6.6 SENSITIVITY AND SPECIFICITY OF THE ZONA-FREE HAMSTER OVUM SPERM PENETRATION ASSAY IN RELATION TO HUMAN *IN VITRO* FERTILISATION

### 6.6.1 Introduction

The right selection of couples for admission to an *in vitro* fertilisation (IVF) and embryo transfer (ET) program is not only the first but also a very important step in the procedure. The screening of the male partner usually relies upon the results of the conventional SA, which gives quantititive data about the semen. The SPA can give additional information on the ability of spermatozoa to capacitate and to penetrate the membrane of a zona-pellucida free oocyte. These are important conditions for fertilisation. There is no agreement in the literature, however, on the correlation between the SPA and male fertility.[8,9,15-22] Furthermore, only few reports have been published about

the correlation between the SPA and human IVF.[5,56-59] These publications show conflicting results.

In order to evaluate the prognostic value of the heterologous zona-free hamster ovum sperm penetration assay, the results of this test were compared with the fertilisation of human ova in a program for IVF and ET. The sensitivity and specificity of the SPA calculated from the data of this study are compared with those calculated from the data of other reports.

### 6.6.2 Materials and methods

#### *6.6.2.1 Patients*

The patient group in this study consisted of 35 couples which entered the IVF program in Nijmegen between May 1984 and October 1985. In all couples IVF was applied to treat tubal infertility and there were no other abnormalities in their routine fertility investigation. All men had a normal SA previous to entering the IVF program. The criteria for normal SA values are given in 6.2.2.2.

#### *6.6.2.2 Collection and preparation of sperm*

The collection and preparation of the semen samples were almost identical for the SPA and for the IVF. In three cases the SPA and the IVF were carried out with aliquots of the same ejaculate. In all other men the SPA was performed approximately one month before the IVF.

After liquefaction, the ejaculate was centrifugated on a 80% Percoll layer to remove abnormal spermatozoa and white blood cells, if present, and then washed twice with BWW medium.

#### *6.6.2.3 SPA*

The SPA was carried out as already described.(6.2.2.3) Only semen samples with a sufficient motility after preincubation (i.e. motility grade ≥4) were considered. When the SPA was negative, the SPA was performed a second time with another semen sample; the highest score was used for this study. A negative SPA means at least two negative SPAs on two different occasions.

#### *6.6.2.4 IVF*

Ovarian hyperstimulation was carried out with human menopausal gonadotrophin (HMG, Humegon®, Organon, Oss, The Netherlands) and human chorionic gonadotrophin (HCG, Pregnyl®, Organon, Oss, The Netherlands), or with the combination of clomiphene citrate (Clomid®, Gist-brocades, Rijswijk, The Netherlands) and HMG/HCG. Follicular growth was monitored by ultrasound in combination with the determination of the ratio oestrogens to kreatinin in early morning urine. When the mean diameter of at least one follicle reached 14 mms and the oestrogen to kreatinin ratio exceeded 68, the HMG injections were discontinued. When three follicles or more reached a diameter of at least 15 mms and the oestrogen to kreatinin ratio exceeded 114,

HCG was administered. Approximately 34 hours after the HCG injection the oocytes were recovered by laparoscopy under general anaesthesia. Only those couples were admitted to the study in which the laparoscopy succeeded in obtaining mature oocytes.

Semen was collected the same morning, centrifugated on a 80% Percoll layer and then washed twice with BWW medium. The spermatozoa were incubated for 4 to 6 hours at 37°C and were then inseminated with the oocytes in a concentration of 25.000 to 50.000/mL. Evidence of fertilisation was the presence of cleavage to the 2-, 4- or 8 cell stage 40 hours after insemination. When not two pronuclei were seen or when the oocytes showed no cleavage on the third day after the laparoscopy the IVF was judged as negative.

*6.6.2.5 Statistics*

The sensitivity is the ability of a test to identify a disease when it is indeed present.[60] It is reflected in the proportion of negative SPAs of those who failed to fertilise in the IVF. The specificity is the ability of a test to identify the absence of a disease when the disease is indeed absent. It is shown in the proportion of positive SPAs of those who succeeded to fertilise human oocytes in the IVF. In a 2 x 2 table the calculation of sensitivity and specificity of the SPA in relation to the outcome of IVF is shown.

**Table VI.12** Comparison of test results and clinical condition

| | pos. disease (= IVF neg.) | neg. disease (= IVF pos.) |
|---|---|---|
| pos. test (= SPA 0%) | a | b |
| neg. test (= SPA >0%) | c | d |

Sensitivity: $\frac{a}{a+c} \times 100\%$

Specificity: $\frac{d}{b+d} \times 100\%$

The positive predictive value of a test is the probability that a positive SPA result is accurate (IVF indeed negative). It is given in the formula a/a+b.

### 6.6.3 Results

The results of the SPA and human IVF were compared in 35 couples (table VI.13).

**Table VI.13** Contingency table for SPA and IVF results

| | IVF negative | IVF positive |
|---|---|---|
| SPA negative | 5 | 6 |
| SPA positive | 4 | 20 |

In 25 of patients concordant results were seen in the SPA and IVF. Five males had both a negative SPA and a negative human IVF. One of these 5 men again failed to fertilise his wife's oocytes on a second IVF attempt. In four cases false positive results (penetration in the SPA and no fertilisation of the human oocytes) were seen. Although the semen of 11 males repeatedly failed to penetrate hamster eggs 6 of them achieved fertilisation of their wife's oocytes which resulted in one pregnancy.

The sensitivity of the SPA in this study was 56% (5/9), whereas the specificity was 77% (20/26). The predictive value of a positive SPA was 83% (20 of 24), whereas 45% (5 of 11) of the men with a negative SPA were able to fertilise human eggs.

The mean penetration rate for the hamster egg penetration assay was not significantly different for men who succeeded and men who failed to fertilise their wife's oocytes (51% vs 45%).

### 6.6.4 Discussion

The main indication for IVF and ET is absent or impaired tubal function. The evaluation of the male fertility in couples asking for IVF is usually based on the results of routine semen analyses. It is well accepted that some men despite normal semen parameters are infertile and that men with proven fertility can have a semen quality well below the normal range. The SPA is an additional parameter with which the sperm function can be assessed.

The aim of this study was to investigate the validity of the SPA as a screening parameter for human IVF. The results of the SPA of males with normal routine semen characteristics were compared with the results of fertilisation and cleavage in a human IVF program. The data showed that the chance of succesfull fertilisation in the human IVF procedure is relatively high both for the males who had a positive SPA as for those with a negative SPA, 83% (20/24) and 55% (6/11), respectively.

In some studies SPA- and IVF results were compared. The different studies on the SPA are difficult to compare due to the lack of standardisation of the technical performance of this assay.[5] Furthermore there is no consistency about the cut-off value of the

penetration rate to distinguish between a positive or a negative SPA. In our laboratory we define a negative SPA as one without penetration. (see 6.2.2.3)

The sensitivity of a test improves as the cut-off point is raised, but as a consequence specificity and predictive value are reduced. Despite the low limit for a positive SPA (0%), the specificity of this assay was less in this study compared to the specificity suggested by other investigators. (table VI.14) The sensitivity in this study, however, was higher than that of other investigators.

**Table VI.14** Validity of the SPA in relation to human IVF as assessed from the results of several authors

| Author | n | Lower limit of hamster egg penetration (%) | Sensitivity | Specificity |
|---|---|---|---|---|
| Wolf et al.[56] | 24 | 10 | 0 | 89 |
| Foreman et al.[57] | 37 | 1 | 40 | 100 |
| Ausmanas et al.[58] | 54 | 15 | 33 | 73 |
| Rogers[5] | 29 | 10 | 50 | 92 |
| Margalioth et al[59] | 82 | 20 | 50 | 100 |
| This study | 35 | 1 | 56 | 77 |

In case of false positive results it is difficult to distinguish between impaired fertilising capacity of the spermatozoa or insufficient quality of the human oocytes, although in this study only IVF treatments were considered in which mature oocytes were collected. The sensitivity of the SPA which identifies the infertile men who are really infertile, was low in all studies. The proportion of men with a negative SPA who succeeded to fertilise one or more human oocytes does not justify the conclusion of some that the SPA is a useful screening parameter for IVF. Apparently the sperm cells can penetrate human oocytes more easily than they can penetrate hamster eggs.

Because of the high incidence of false negative and false positive results of the SPA in relation to IVF results and also because of the impractability of the SPA, this assay seems to be of limited value in selecting male partners of particular couples seeking IVF/ET. In case of normal routine semen analysis the SPA is no addition or replacement to predict the success of human IVF.

## 6.7 GENERAL DISCUSSION AND CONCLUSIONS ON THE ZONA-FREE HAMSTER OVUM SPERM PENETRATION ASSAY

Male infertility or subfertility can be caused by a variety of factors. In most of the cases the disorder can be confirmed by abnormalities of the semen. Routine SA is the most widely used method to assess a male's fertility potential. SA gives objective, quantitative data. A clear prognosis of an individual case cannot be made on the basis of data obtained from the SA only. The functional competence of the spermatozoa is not necessarily reflected in the conventional semen profile.

The SPM test and the SPA are two additional assays which evaluate the functional capacity of spermatozoa to penetrate cervical mucus and to penetrate the oocyte membrane of a zona-pellucida free hamster egg. Both assays simulate processes that occur *in vivo*. In the SPM test both the quality of the cervical mucus and of the sperm cells are determined. By using donor cervical mucus the SPM test can provide objective information on the motility of the spermatozoa.

The SPA is a laborious, not routinely used assay. This test simulates the last events just prior to the actual fertilisation. It is a great disadvantage that the technical procedure of the test is not standardised and that the outcome of the SPA is dependent on many variables. Before the sperm cells are exposed to the hamster eggs they have to undergo capacitation and acrosome reaction. This process takes place *in vitro* while the spermatozoa are washed from the semen plasma and preincubated in a medium. The results of the SPA are still equivocal, since sperm from some infertile men do penetrate zona-free hamster oocytes and, conversely, spermatozoa from some fertile men do not. The potential usefulness of this technique in fertility investigations requires further research.

In this chapter studies are mentioned which describe the correlations between the SPA and other tests of semen quality and the influence of variables in the technical procedure on the results of the SPA.

It was shown that a positive correlation existed between the parameters of the routine SA and the outcome of the SPA. A normal semen parameter gave a higher chance of a positive SPA and the more normal characteristics of the routine SA were seen, the higher the chance of a positive SPA. However, there were still a number of possibly infertile men who had a negative SPA despite normal routine SA. For an individual male it is not possible to predict the outcome of the SPA.

When the motility after the preincubation period was taken into account in the interpretation of the SPA result, the number of men who scored negative in the SPA despite normal semen characteristics, decreased. Most likely only those men with repeatedly negative SPAs and sufficient motility after preincubation are really infertile.

Another variable that influences the outcome of the SPA is the presence of white blood cells in the ejaculate. White blood cells have a negative effect on the fertilisation potential of spermatozoa. Centrifugation over a Percoll-layer of the semen sample can separate the spermatozoa from other components in the semen. However, the Percoll

washing procedure showed no advantage on the SPA results or on the motility grade after preincubation. It is likely that the white blood cells have exerted their effect on the spermatozoa before the washing procedure.

The SPA and SPM test are both functional tests which determine sperm quality, in contrast with the routine SA which is a quantitative test. Positive correlations were seen between the SPM score and both the percentage of men with a positive SPA and the penetration rate in the SPA. Although a positive correlation between these two tests was observed, the range of the SPA result was too large to predict the outcome of the SPA on the base of the SPM score for an individual male. It is likely that the SPA and the SPM test measure different sperm properties, and that both tests contribute separately to the total fertility investigation of a man. Not all events of fertilisation can be examined by the SPA, but the existence of correlations between the outcome of the SPA and other aspects of sperm function including the penetration of cervical mucus, support the view that such tests may be of value in evaluating male fertility.

The validity of the SPA was assessed by comparing the results of the SPA with the fertilisation of human oocytes in a program for IVF and ET. Both the sensitivity and the specificity of the SPA were relatively low to use this assay in selecting male partners for IVF and ET.

The diagnostic value of the SPA has to be established further by performing carefully designed prospective studies. However, a major disadvantage of the evaluation of the fertility potential of an individual is the variability between ejaculates.

**Final conclusions:**

Neither routine SA, nor the SPA are absolute criteria for fertility. Routine SA is a quantitative- and the SPA is a qualitative-assay, but this latter assay does not examine the spermatozoon's ability to attach itself or to penetrate the zona pellucida. For this reason a positive result of the SPA should be interpreted with caution and such a result does not necessarily reflect that spermatozoa are capable to fertilise human oocytes *in vivo* or *in vitro*.

A negative SPA should also be evaluated cautiously. Many non-standardised technical variables influence the outcome of the SPA. This means that a negative SPA does not always preclude the possibility of establishing a pregnancy, especially when the motility after the preincubation period is not optimal.

The only valid indication for a SPA is unexplained infertility and sufficient motility after the preincubation period. Therefore the indications for the SPA should be limited to semen samples with sufficient motility after preincubation.

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# CHAPTER 7

# GENERAL DISCUSSION

Approximately one in ten couples do not succeed in establishing a pregnancy after one year of unprotected intercourse. The number of couples seeking medical help for infertility is increasing. This stresses the importance of careful and accurate investigation of a couple's infertility.

In many instances the main examinations and tests of the fertility workup program (see 1.3) reveal abnormalities as possible cause of infertility, and thereby indicating a rational therapy. The majority of infertile patients, however, do not have unequivocal findings and should be considered 'subfertile'. The routine fertility workup program of a number of couples reveal no abnormalities that can explain their infertility. This investigation has been performed to study carefully the usefulness and completeness of some steps in the routine fertility workup program.

The fertility potential of a couple depends on the normalities of three distinct steps in reproduction:

1. Gamete production
2. Fertilisation: gamete fusion
3. Implantation

In the previous chapters several aspects of these three steps have been studied and discussed.

In the female an extensive investigation of the menstrual cycle has been performed, both in fertile and infertile patients. Frequent sampling of blood throughout one menstrual cycle provides information on cyclical patterns regulated through endocrine mechanisms. It was observed that prolactin does not demonstrate a definite cyclical pattern, but that the values of this hormone fluctuate throughout the cycle. Intermittent elevations of prolactin can be demonstrated, both in fertile and infertile women with either irregular or regular menstrual cycles. However, prolactin levels in the fertile group were only occasionally moderately above the level of normal prolactin concentrations, whereas in the infertile group several women showed more frequently clearly elevated prolactin concentrations. These patients can be considered as having transient hyperprolactinaemia. The frequent, rather high elevations of prolactin levels in infertile patients suggest interference of this hormone with their fertility potential. The way transient hyperprolactinaemia interferes with the regulation of the menstrual cycle is not clear. It is advisable to perform prolactin measurement in a fertility workup program in case of anovulation and/or amenorrhoea. This investigation stresses the need of measurement of prolactin at least twice to exclude transient hyperprolactinaemia, independent of whether the woman has a regular or irregular cycle.

The causes of menstrual irregularity are various and are mostly attended by anovulation. The infertile women of this study all demonstrated definite signs of ovulation. The endocrinological parameters of these women strongly indicated a mild or early form of PCOD. The PCO-syndrome is an ill defined disorder and a large clinical and biochemical variability is encountered in this disease. The main symptoms associated with PCOD are anovulation, signs of androgen excess and obesity. The endocrinological characteristics of PCOD patients are elevated LH levels, normal or decreased FSH levels, resulting in an increased LH to FSH ratio, and elevated androgen and oestrogen values. The infertile women with irregular cycles in this investigation showed signs of inappropriate gonadotrophin secretion and elevated concentrations of androgenic steroids. Especially the saliva androgens, which are supposed to represent the free proportion of the hormone, were found to be elevated in many women with irregular cycles. This was also the case in the group with unexplained infertility.

Despite ovulatory cyles, and even regular cycles, several endocrinological abnormalities have been demonstrated in the infertile women. These findings indicate the usefulness of measuring gonadotrophins and saliva testosterone in all infertile women, also in those with ovulatory cycles.

In studying the fertility of the male, SA is the most widely used and also easiest method. Several other, more recently developed tests are available to investigate the fertility potential of men, such as the SPM test and the SPA. All tests show a wide range of results, both in the fertile and in the infertile males. A large overlap exists in values obtained from fertile and infertile men. The results of the SA, SPM-test and SPA are not always in accordance with each other. Therefore it is likely that these tests measure different properties of the spermatozoa. The SA, SPM-test and SPA are complementary in the investigation of male fertility.

It should be emphasized that the motility after the preincubation period of the spermatozoa, i.e. the period in which the spermatozoa have to undergo acrosome reaction and capacitation, is an important variable which determines the outcome in the SPA. Spermatozoa with an insufficient motility after preincubation are less capable to penetrate zona-free hamster oocytes. Therefore a negative SPA in combination with insufficient motility after preincubation does not per se reflect an intrinsic defect in sperm fertilising function. The results of the SPA and human IVF have been compared. The relatively low correlation between the SPA and the results in human IVF also stresses the need of a careful interpretation of the SPA results. The SPA is of limited value in measuring the fertilising ability of a semen sample.

Implantation, the third event in normal reproduction, is difficult to investigate, without interfering with a pregnancy. One possibility to study this process is by determination of hCG, the hormone that is secreted at a very early stage by the conceptus, and that can be detected from the time of implantation onwards. Based on hCG determinations only, failure of implantation, early conceptual loss, is probably a normal event in the human reproductive process. By measuring hCG frequently in a prospective way both in fertile

women and in women with unexplained infertility, it was found that early conceptual loss is not likely to occur more often in infertile women, and is not a significant cause of unexplained infertility. Until now, no method is available to detect conceptual loss before implantation after normal *in vivo* fertilisation. Such a method would not only be helpful in detecting conceptual loss in infertile patients, but also in the investigation of successes and failures in human IVF and ET.

The observation that 90% of the couples with unexplained infertility showed one or more subtle abnormalities emphasisis the importance of an accurate investigation of a couple's infertility. The diagnosis 'unexplained infertility' is only justified when also endocrinological abnormalities, such as transient hyperprolactinaemia and excess of androgen production, are excluded and an extensive investigation of the ejaculate, including at least three SAs and a SPA, has been performed. Human IVF would be of diagnostical help in the evaluation of both follicular development, oocyte maturation, male spermatozoal quality and the ability of fusion of male and female gametes.

Although it is tempting to attribute the described abnormalities to the cause of infertility, similar abnormalities have been observed in fertile couples. The difference between fertile and infertile couples involves the frequency of subtle abnormalities, and the combination of male and female abnormalities. An abnormality of one partner can probably be compensated by very good fertility parameters of the other partner of a couple. Furthermore, a suboptimal cycle or suboptimal ejaculate may subsequently be followed by a normal one. Abnormalities do not appear to be repetitive, this also explains why many women with either explained or unexplained infertility become pregnant spontaneously after a period of time.

Conscientiously designed, prospective clinical trials are necessary to study the need for treatment in cases with 'subtle endocrinological abnormalities'. The results of treatment should be compared to the frequency of pregnancies which are treatment-independent.

# SUMMARY

Approximately ten percent of all couples trying to conceive do not succeed in establishing a pregnancy within one year. Both male and female factors and a combination of these can be responsable for the infertility. The standard investigation of a couple does not always reveal the cause of infertility, around five to ten percent of infertility is considered as unexplained. In detecting the cause of infertility the extensiveness of the fertility workup program is crucial.

The studies presented in this thesis concern some newly developed diagnostic methods which are not included in the routine investigation of infertility.

In the introduction the physiology of reproduction is reviewed. The process of normal fertilisation and implantation is described and the current concepts of the endocrinological events during the menstrual cycle and the role of androgenic steroids are summarised. An outline of the basic investigation of infertility is given.

The first part of this thesis is a prospective, extensive study of the menstrual cycle. Three groups participated in the study: a group of 15 infertile women with irregular menstrual cycles, a group of 20 women with unexplained infertility and 20 assumed fertile women, who formed the control group.

A description is given of the selection criteria, clinical characteristics and semen parameters of the groups, the schedule of blood and saliva collection and the immunochemical procedures are presented and the statistical methods applied are explained.

The endocrinological parameters of the women with irregular cycles and of those with unexplained infertility are compared with those of the control group.

The LH levels and the LH/FSH ratios of the group with irregular cycles tended to be higher. Furthermore serum testosterone levels and saliva androgens were in a higher range in the women with irregular cycles. It is supposed that women with irregular but ovulatory cycles suffer from a mild or early form of PCOD.

The LH concentrations of the women with unexplained infertility were higher during the follicular phase. The 17ß-oestradiol levels in serum as well in saliva were elevated, probably as aresult of the increased LH levels. The higher follicular LH levels can cause infertility by early luteinisation of the follicle or by a direct effect of LH on oocyte maturation.

In all groups a large variation was seen in prolactin levels both within one subject as well as between subjects. In the infertile women some could be assigned as suffering from transient hyperprolactinaemia with frequently elevated prolactin concentrations during the study cycle. It is concluded that at least two prolactin determinations are required in all infertile women.

Eighteen of the 20 couples with unexplained infertility showed one or more subtle abnormalities in the female's hormonal secretory pattern and/or in the male's investigation of the ejaculate after extensive investigation. This suggests that one should be very careful with the diagnosis of unexplained infertility.

In the second part of the study hCG was measured during the luteal phase of 20 assumed fertile women trying to conceive and in 20 women with unexplained infertility to detect conception at an early stage. Five clinical, ongoing pregnancies were observed in the fertile group. The range in first day of detection of hCG in these women was high. This indicates the need for very cautious interpretation of luteal hCG concentrations. Based on hCG determinations in the luteal phase clinically unrecognised, early conceptual loss after implantation forms no major explanation for unexplained infertility.

The last part of the thesis deals with semen parameters, especially the zona-free hamster ovum sperm penetration assay. The relation between the SPA and the routine semen analysis was studied in a group of 220 males of infertile couples. Every normal semen parameter showed a statistically significantly higher chance to contribute to a positive SPA compared with an abnormal semen parameter. Furthermore, the more normal semen parameters in the SA were present, the more males showed a positive SPA. In the study group as a whole a significant correlation was demonstrated between the SA and the SPA, but the large variation in SPA results reflect the inability to predict the SPA outcome of an individual.

This study demonstrated a statistically significant decrease inthe number of positive SPA scores when the motility grade of the spermatozoa after the preincubation period is insufficient. Motility after preincubation showed a stronger correlation with the SPA than any of the other routine semen characteristics.

The presence of white blood cells in the ejaculate had a negative effect on sperm function as reflected in the outcome of the SPA, From an experiment with centrifugation of the semen sample over a Percoll layer to remove white blood cells, compared with the routine BWW washing method, it was concluded that the white blood cells had their deterious effect on the spermatozoa already before separation.

Positive correlations were observed between the SPM-score and both the percentage of men with a positive SPA and the penetration rate in the SPA.

In an attempt to compare the SPA with the actual fertilisation in human *in vitro* fertilisation, the data showed that the chance of succesful *in vitro* fertilisation was relatively high both for the males who had a positive SPA as for those with a negative SPA. It was therefore concluded that the SPA cannot be used as a screening procedure for IVF.

Carefully designed, prospective, basal and clinical investigations remain necessary to evaluate which factors do and which factors do not actually attribute to a couple's infertility.

# SAMENVATTING

Ongeveer 10% van de paren met kinderwens slaagt er niet in deze wens te vervullen binnen één jaar onbeschermde coitus. Zowel mannelijke als vrouwelijke factoren, of een combinatie, kunnen onvruchtbaarheid veroorzaken. Het standaard vruchtbaarheidsonderzoek toont niet altijd de reden van de onvruchtbaarheid, naar schatting 5 tot 10% van de onvruchtbaarheid wordt beschouwd als onverklaard. De uitvoerigheid van het vruchtbaarheidsonderzoek is bepalend bij het opsporen van de oorzaak van de onvruchtbaarheid.

De studies in dit proefschrift hebben betrekking op enkele recent ontwikkelde diagnostische methoden die niet tot het standaard onvruchtbaarheidsonderzoek behoren.

In de inleiding wordt een overzicht gegeven van de physiologie van de voortplanting. Het proces van de normale bevruchting en innesteling wordt beschreven en de huidige inzichten met betrekking tot de hormonale veranderingen van de menstruele cyclus en de rol van de androgene steroiden worden besproken. Een samenvatting van het standaard onvruchtbaarheidsonderzoek wordt gegeven.

Het eerste deel van het proefschrift omvat een uitgebreid, prospectief onderzoek naar de menstruele cyclus. Drie groepen namen deel aan de studie: een groep van 15 onvruchtbare vrouwen met onregelmatige cycli, een groep van 20 vrouwen met onverklaarde onvruchtbaarheid en 20 vrouwen van wie werd verwacht dat zij vruchtbaar waren. Deze laatste groep vormde de controle groep.

Er wordt een beschrijving gegeven van de selectie-criteria, de klinische kenmerken en de semen gegevens van de groepen, het schema van het verzamelen van de bloed- en speeksel monsters, de bepalingstechnieken en de statistische methoden.

De hormonale gegevens van de vrouwen met onregelmatige cycli en die van de vrouwen met onverklaarde onvruchtbaarheid worden beiden vergeleken met de gegevens van de controle groep.

De spiegels van LH en de LH/FSH ratio's van de groep met onregelmatige cycli vertoonden een tendens naar hogere waarden. Bovendien neigden de serum testosteron en speeksel androgenen spiegels naar hogere waarden bij de vrouwen met onregelmatige cycli. Er wordt verondersteld dat vrouwen met onregelmatige, maar ovulatoire cycli leiden aan een milde of vroege vorm van het polycysteus ovarium syndroom.

De LH concentraties van de vrouwen met onverklaarde onvruchtbaarheid waren hoger tijdens de folliculaire fase. De 17β-oestradiol spiegels zowel in serum als in speeksel waren verhoogd, waarschijnlijk als gevolg van de toegenomen LH concentraties. De hogere LH spiegels kunnen onvruchtbaarheid veroorzaken door vroege luteinisatie of door een direct effect van LH op de rijping van de eicel.

In alle groepen werd een grote spreiding gezien in de waarden van prolactine, zowel tussen de waarden van de cyclus van één vrouw, als tussen de waarden van de cyclus van verschillende vrouwen. Bij sommige vrouwen uit de onvruchtbare groepen kan de diagnose 'intermitterende hyperprolactinaemie' worden gesteld, omdat bij hen herhaal-

delijk verhoogde prolactine concentraties werden gemeten gedurende de onderzoekscyclus. Geconcludeerd wordt dat bij alle onvruchtbare vrouwen tenminste twee maal het prolactine gehalte moet worden bepaald.

Bij 18 van de 20 paren met onverklaarde onvruchtbaarheid werden één of meerdere kleine afwijkingen gevonden in het hormonale patroon van de vrouw en/of in het uitgebreide semen onderzoek van de man. Dit duidt aan dat de diagnose 'onverklaarde onvruchtbaarheid' zorgvuldig moet worden gehanteerd.

In het tweede deel van het onderzoek wordt getracht zeer vroeg te bepalen of bevruchting heeft plaats gevonden. HCG werd gemeten tijdens de luteale fase van de cyclus van 20 vruchtbare vrouwen die zwangerschap nastreefden en 20 vrouwen met onverklaarde onvruchtbaarheid. In de vruchtbare groep werden 5 klinische, doorgaande zwangerschappen waargenomen. Er was een grote spreiding in het tijdstip waarop voor de eerste maal hCG kon worden gemeten in het serum van deze vrouwen. Dit toont aan dat hCG waarden in de luteale fase voorzichtig moeten worden geïnterpreteerd. Klinisch onherkend, vroeg verlies van de conceptus na de innesteling, gebaseerd op hCG bepalingen in de luteale fase, is geen belangrijke oorzaak voor onverklaarde onvruchtbaarheid.

Het laatste gedeelte van het proefschrift gaat in op het onderzoek van semen, in het bijzonder de hamstereiceltest. De relatie tussen de hamstereiceltest en de traditionele semen analyse werd bestudeerd in een groep mannelijke partners van 220 onvruchtbare paren. Elke normale semen parameter afzonderlijk had een statistisch significant hogere kans om bij te dragen aan een positieve hamstereiceltest dan een abnormale semen parameter. De kans op een positieve hamstereiceltest nam toe naarmate meer normale semen karakteristieken werden gezien bij de semen analyse. In de onderzoeksgroep als geheel werd een significante correlatie aangetoond tussen de semen analyse en de hamstereiceltest, maar de grote spreiding in hamster eicel test uitkomsten weerspiegelde het onvermogen van de semen analyse om de uitkomst van de hamstereiceltest van een bepaalde persoon te voorspellen.

Deze studie toont aan dat het aantal positieve hamstereiceltesten significant afneemt indien de beweeglijkheid van de zaadcellen na de pre-incubatietijd onvoldoende is. De beweeglijkheid van de zaadcellen na de pre-incubatietijd had een sterkere correlatie met de hamstereiceltest dan elk van de andere traditionele semen karakteristieken.

De aanwezigheid van witte bloedcellen in het ejaculaat had een negatief effect op de funktie van de zaadcellen, zoals gemeten in de hamstereiceltest. Uit een onderzoek waarbij centrifugatie van het zaad monster met een Percoll oplossing, om eventuele witte bloedcellen te verwijderen, werd vergeleken met de traditionele BWW methode, werd geconcludeerd dat de witte bloedcellen hun negatief effect op de spermatozoa al hadden uitgeoefend voor de scheiding.

Positieve correlaties werden aangetoond tussen de SPM-score en zowel het percentage mannen met een positieve hamstereiceltest als de penetratie graad in de hamstereiceltest.

In een poging om de hamster eicel test te vergelijken met de feitelijke bevruchting in *in vitro* fertilisatie bij mensen, werd uit de gegevens van 35 paren aangetoond dat de kans op succesvolle *in vitro* fertilisatie relatief hoog was, zowel voor de mannen met een positieve als met een negatieve hamstereiceltest. Daarom werd geconcludeerd dat de hamstereiceltest geen goede selectie methode is voor *in vitro* fertilisatie.

Zorgvuldig opgezette, prospectieve, basale en klinische onderzoeken blijven noodzakelijk voor de evaluatie van de factoren welke wel en welke niet feitelijk bijdragen aan de onvruchtbaarheid van een paar.

# ABBREVIATIONS

| | |
|---|---|
| ACTH | AdrenoCorticoTrophic Hormone |
| Andr | Androstenedione |
| BBT | Basal Body Temperature |
| BWW | Biggers-Whitten-Whittingham medium |
| Cor | Cortisol |
| DHEA | DeHydroEpiAndrosterone |
| DHEAS | DeHydroEpiAndrosterone Sulphate |
| ET | Embryo Transfer |
| FAI | Free Androgen Index |
| FSH | Follicle Stimulating Hormone |
| GnRH | Gonadotrophin Releasing Hormone |
| hCG | Human Chorionic Gonadotrophin |
| hGH | Human Growth Hormone |
| HMG | Human Menopausal Gonadotrophin |
| hPL | Human Placental Lactogen |
| IEMA | ImmunoEnzyMetric Assay |
| IRMA | ImmunoRadioMetric Assay |
| IRP | International Reference Preparation |
| IS | International Standard |
| IVF | In Vitro Fertilisation |
| LH | Luteinising Hormone |
| LHRH | Luteinising Hormone Releasing Hormone |
| MAP | Motility After Preincubation |
| MAR | Mixed Antiglobulin Reaction test |
| OE2 | 17ß-oestradiol |
| PCOD | PolyCystic Ovarian Disease |
| PRL | Prolactin |
| Prog | Progesterone |
| RIA | Radio ImmunoAssay |
| SA | Semen Analysis |
| SD | Standard Deviation |
| SEM | Standard Error of the Mean |
| 17OHP | 17α-OH-Progesterone |
| SHBG | Sex Hormone Binding Globulin |
| SPA | Sperm Penetration Assay |
| SPM | Sperm Penetration Meter test |
| $T_4$ | Thyroxine |
| Test | Testosterone |
| WBC | White Blood Cell |

# Curriculum vitae

Dyonne van Duren werd geboren op 5 juli 1956 in Handel (Noord-Brabant). Zij behaalde in 1974 haar Atheneum-B diploma aan het Thomas a Kempiscollege in Arnhem.
Zij studeerde één jaar economie aan de Rijksuniversiteit te Groningen.
Van 1975 tot 1982 studeerde zij geneeskunde aan de Katholieke Universiteit te Nijmegen. Tijdens de doctoraalfase van deze studie werkte zij als student-assistent mee in het projekt "Gasanalyse van navelstrengbloed bij bevallingen thuis" onder leiding van Prof.Dr. T.K.A.B. Eskes. Na haar doctoraalexamen, in maart 1980, verrichtte zij samen met mw. Drs. E.W.M. Spanjaards een wetenschappelijke stage "Obstetrische aspekten van de zwangerschap ontstaan tijdens anti-oestrogenen therapie", begeleid door Prof.Dr. R. Rolland. Zij behaalde haar arts-examen op 21 mei 1982.
Van juni 1982 tot oktober 1983 was zij als arts-assistent werkzaam op de afdeling Gynaecologie\Verloskunde van het Sophiaziekenhuis in Zwolle (B-opleiding, opleider Dr. F. Engel).
Van november 1983 tot november 1987 was zij wetenschappelijk assistent op de afdeling Gynaecologie\Verloskunde van het St. Radboudziekenhuis in Nijmegen. In het kader van een "Universitair Onderzoeks Pool"-projekt werd een promotie-onderzoek financieel mogelijk gemaakt door de faculteit geneeskunde. Vanaf 1 april 1988 is zij in opleiding tot specialist in de Verloskunde en Gynaecologie in het St. Radboudziekenhuis te Nijmegen.
De auteur is gehuwd met Theo van Buren, leraar geschiedenis en staatsinrichting, en moeder van Jan-Willem van 2,5 jaar en Diderik van 9 maanden.

Stellingen behorende bij het proefschrift

# THE INFERTILE COUPLE:
## SOME CYCLE AND SPERM CHARACTERISTICS

door

**Bernardina Petronella Johanna van Duren**

# STELLINGEN

1 Bij iedere vrouw die een fertiliteitsonderzoek ondergaat, moet de prolactine concentratie minstens twee maal worden bepaald. (dit proefschrift)

2 Het speeksel testosteron gehalte is een waardevolle parameter in het fertiliteitsonderzoek van de vrouw. (dit proefschrift)

3 Het verrichten van een hamstereiceltest is alleen zinvol indien de motiliteit van de spermatozoa na voorbewerking voldoende is. (dit proefschrift)

4 Het dichterbij brengen van de zaadcel bij de eicel betekent niet noodzakelijk dat de kans op zwangerschap toeneemt.

5 Psychogene infertiliteit is niet bewezen.

6 Aangezien Homerus in παρευναζομαι geen trema gebruikt is het onlogisch dat in dyspareunie wel te doen.

7 Bevallen is een goed begin van de opleiding tot vrouwenarts.

8 Stellingen verhogen niet de wetenschappelijke waarde van een proefschrift.